THE ROLE OF EPIDERMAL GROWTH FACTOR RECEPTOR 2 OVEREXPRESSION ON ATTENUATING REACTIVE OXYGEN SPECIES IN THE HEART

by
Frances R. Belmonte

A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland
June, 2014

Abstract

Current statistics for the incidence of breast cancer reveal that it is the most common form of cancer and leading cause of death from cancer among women worldwide. In the US, breast cancer follows lung cancer as the second largest cause of cancer mortality and is the most common type of cancer in American women. Approximately 15-20% of breast cancers are characterized as HER2/neu/ErbB2 (epidermal growth factor receptor 2)-positive. The combination of doxorubicin and the ErbB2 inhibitor Herceptin/trastuzumab is efficacious against ErbB2-overexpressing breast cancer but has been shown to cause cardiotoxicity and has been linked to an increased risk for developing cardiac failure.

The following chapters of this dissertation will explore the intricacies of ErbB2 tyrosine kinase signaling and the essential roles of ErbB2 in cardiac development and function. For our studies we employed a transgenic murine model of cardiac-specific ErbB2 overexpression developed in our lab that has concentric cardiac hypertrophy that does not progress to heart failure. Since oxidative stress mediates the transition from cardiac hypertrophy to heart failure, we sought to investigate the underlying mechanisms of ErbB2 overexpression on redox signaling pathways involved in antioxidant protection against reactive oxygen species (ROS). Indeed, hearts from the ErbB2 transgenic ($ErbB2^{tg}$) mice and their cardiac mitochondria have lower levels of ROS. We proposed that ErbB2 regulates the Abelson nonreceptor tyrosine kinases to subsequently activate glutathione peroxidase (GPx) and reduce ROS levels. *In vitro* models of ErbB2 overexpression and abrogration in the H9c2 rat cardiomyoblast cell line were developed to assess these associations. Studies with neonatal cardiomyocytes suggest that GPx

activity in the ErbB2^{tg} mice is dependent upon the hypertrophic phenotype that is conspicuously absent in the H9c2 cells.

Using the ErbB2^{tg} model we identified a unique multi-protein signaling pathway that supports a cardioprotective role of ErbB2 in reducing ROS levels in the heart. It is possible that ErbB2 inhibitors can effectively block these components that are necessary for preventing adverse cardiac outcomes. The ErbB2^{tg} mouse model is a valuable tool for the discovery of novel mechanisms and signaling pathways that have potential translational significance for cardiovascular studies and cancer biology.

Advisor: Kathleen Gabrielson, DVM, PhD

Readers: Joseph Bressler, PhD

Valeria Culotta, PhD

Nazareno Paolocci, MD, PhD

Acknowledgements

I would like to acknowledge Dr. Kathleen Gabrielson and all of the lab members—past and current—who provided their guidance and assistance throughout the years. I am grateful for their knowledge and expertise and am fortunate to have acquired an enormous amount of skills and techniques that will be valuable for all of my future research endeavors.

Past and current lab members (in no particular order): Dr. Sysa-Shah, Dr. Yi Xu, Dr. Xin Guo

I would next like to acknowledge the thesis committee and thank them for their time reviewing the dissertation and for their constructive feedback during my committee meetings:

Dr. Joseph Bressler

Dr. Valeria Culotta

Dr. Nazareno Paolocci

Dr. Charles Steenbergen

There are many individuals to whom I owe my sincere gratitude over the past several years so rather than making a lengthy list of names, I would like to acknowledge all of the teachers, mentors, advisors, postdocs, and colleagues who have helped steer me in the direction of what I hope will become a life-long career in research. I am continuously inspired by their intellectual curiosity and enthusiasm.

I am especially thankful for the mentorship that I received from Dr. Sam Das, who has helped me in countless ways and who serves as a role model for all those aspiring to pursue a career in academia.

I would like to extend my gratitude to the EHS department and administrative staff (EHS and Molecular and Comparative Pathobiology) for their assistance with my graduate requirements and for keeping me well-informed about my responsibilities towards completing my degree.

Finally, I would like to thank my family and close friends for their support throughout my graduate career. This dissertation is especially dedicated to my older siblings Christopher and Evita, who I doubt will ever read this but are nonetheless my biggest critics and even bigger supporters.

Table of Contents

List of Tables

Chapter 4: Characterization of cardiac mitochondria isolated from the ErbB2^{tg} mice

List of Figures

Chapter 1: Introduction

Chapter 2: Cardiac-specific overexpression of ErbB2 induces pro-survival pathways and hypertrophic cardiomyopathy in mice

Chapter 3: Overexpression of Epidermal Growth Factor Receptor 2 (ErbB2) in the Heart Alters Redox-Sensitive Molecules that Attenuate Oxidant Stress

Chapter 5: Conclusions and additional studies

List of Abbreviations

Abl	Abelson
ADP	adenosine diphosphate
AKT	protein kinase B
AMPK	adenosine monophosphate-activated protein kinase
Arg	Abelson-related gene
ATP	adenosine triphosphate
Bcl2	b cell lymphoma 2
Bcl-xl	b cell lymphoma-extra large
Bcl-xs	b cell lymphoma-extra short
β2-AR	beta 2- adrenergic receptor
Ca^{2+}	calcium
c-Abl	cytoplasmic Abelson
CaCl2	calcium chloride
$[Ca^{2+}]_m$	mitochondrial matrix calcium
CCCP	carbonyl cyanide *m*-chlorophenyl hydrazone
cDNA	complementary deoxyribonucleic acid
Chk	Csk family tyrosine protein kinase
CMH	1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl pyrrolidine
C_t	threshold cycle
DCF-DA	2,7-dichlorodihydrofluorescein diacetate
ddH_2O	double-distilled water

DMEM	Dulbecco's Modified Eagle's medium
DNP	2,4-dinitrophenol
DNPH	2,4-dinitrophenylhydrazine
dox	doxorubicin
DTNB	5,5-dithio-bis (2-nitrobenzoic acid)
DTPA	diethylene triamine pentaacetic acid
ECD	extracellular domain
EDTA	Ethylenediaminetetraacetic acid
EGFR	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
EPR	electron paramagentic resonance
ErbB2, 3, 4	epidermal growth factor receptor 2, 3, 4
ErbB2^{tg}	ErbB2 transgenic
ERR	estrogen-related receptor
FBS	foetal bovine serum
FDA	Food and Drug Administration
fdr	false discovery rate
g	gravitational
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GHz	Gigahertz
GPx	glutathione peroxidase
GR	glutathione reductase
Grb	growth factor receptor-bound protein

GSH	glutathione
H&E	hematoxylin and eosin
H_2O_2	hydrogen peroxide
HB-EGF	heparin-binding epidermal growth factor-like growth factor
HBSS	Hank's balanced salt solution
HCl	hydrogen chloride
HCM	hypertrophic cardiomyopathy
HEK	human embryonic kidney
HER2	human epidermal growth factor receptor 2
ICRD	intracellular regulatory domain
K_2HPO_4	dipotassium phosphate
KCl	potassium chloride
kg	kilogram
lapa	lapatinib
LVEDd	left ventricular end diastolic diameter
LVESd	left ventricular end systolic diameter
µg	microgram
mg	milligram
ml	milliliter
mmol	millimole
mM	millimolar
µM	micromolar
$\Delta\psi$	mitochondrial membrane potential

MAPK	mitogen-activated protein kinase
MCU	calcium uniporter
MEM	minimum essential media
Mg^{2+}	magnesium
$MgCl_2$	magnesium chloride
αMHC	alpha-myosin heavy chain
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
ms	millisecond
mtDNA	mitochondrial deoxyribonucleic acid
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide
Na_2HPO_4	sodium phosphate dibasic
NAC	N-acetylcysteine
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NaH_2PO_4	monosodium phosphate
Neu	neuregulin
NGF	nerve growth factor
nM	nanomolar
nm	nanometer
NRF	nuclear respiratory factor
NRG-1β	neuregulin-1 beta
nRTKs	nonreceptor tyrosine kinases

NRVM	neonatal rat ventricular myocytes
O_2	oxygen
$O_2^{-\cdot}$	superoxide radical
pAKT	phosphorylated protein kinase B
PBS	phosphate buffered saline
pcDNA	control plasmid
PDGF	plate-derived growth factor
PGC-1	peroxisome proliferator-activated receptor-gamma coactivator-1
PI3K	phosphoinositide 3-kinase
PMSF	phenylmethylsulfonyl fluoride
PPARγ	peroxisome proliferator-activated receptor-gamma
PPIA	peptidylprolylisomerase A
qRT-PCR	quantitative real-time polymerase chain reaction
RaM	rapid-mode calcium uptake
RCR	respiratory control ratio
RNA	ribonucleic acid
R-neu	rat-neuregulin
ROS	reactive oxygen species
RPM	revolutions per minute
RTKs	receptor tyrosine kinases
RyR	ryanodine receptor
Shk	SH2 domain-bearing protein kinase
siRNA	small interfering ribonucleic acid

Src	sarcoma
TCA	tricarboxylic acid
Tfam	mitochondrial transcription factor A
TG	transgenic
TGF-α	transforming growth factor alpha
TKD	tyrosine-kinase domain
TMD	transmembrane domain
TMRE	tetramethylrhodamine ethyl ester
TrxR2	thioredoxin reductase 2
Tyr	tyrosine
U	Unit
UCP	uncoupling protein
veh	Vehicle
WT	wild type
Y	phosphotyrosine residue

Chapter 1

Introduction

1.1 Epidermal growth factor receptor family

The epidermal growth factor (EGF) was initially discovered in 1962 by Stanley Cohen, who was later awarded the 1986 Nobel Prize in Physiology or Medicine [1, 2] along with his colleague Rita Levi-Montalcini, who was credited with Cohen for their joint discovery of the nerve growth factor (NGF) protein [2]. More than two decades following Cohen's seminal discovery, EGFR was identified to contain similar peptides and amino acid sequences as the avian erythroblastosis tumor virus that is encoded by the v-*ErbB*-*B* oncogene, for which the ErbB notation is derived [3]. The ErbB family of receptor tyrosine kinases (RTKs) consists of four members: EGFR, ErbB2 (Her2/neu), ErbB3, and ErbB4[4, 5]. ErbB1 is known to bind to six ligands: EGF, transforming growth factor alpha (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, epiregulin, and β-cellulin [4, 5]. A variety of ligands are known to bind to their corresponding ErbB receptor, such as neuregulins that preferentially bind to ErbB3 and ErbB4. ErbB2, however, has no known soluble ligand but may become activated in the presence of another ErbB receptor-ligand complex[4, 5]. The shared structural features of the ErbBs include cysteine-rich moieties in the extracellular region and tyrosine autophosphorylation sites in the carboxy-terminal tail that is proximal to the kinase domain [5].

An essential element of the ErbB signaling pathway is the different receptor dimers that may form; 10 possible receptor combinations exist among the four ErbB receptors[4, 5]. Since ErbB2 lacks an endogenous ligand, it is the preferred co-receptor for the remaining three receptors [6-9]. As a consequence for the preferential binding to ErbB2, signaling from heterodimer complexes that contain ErbB2 is augmented and stronger

than homodimers. Possible reasons for the potency in ErbB2 signaling include reduced ligand dissociation from the paired receptor and decreased ErbB2 endocytosis [4].

Signaling pathways that are activated by ErbB receptors can be described in terms of the input (i.e. ligands) and the ensuing output from the formation of ligand-receptor hetero- or homodimers [5]. The downstream and intermediate signaling components of these ErbB-driven pathways consist of a wide array of kinases. Examples of these include the mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), and protein kinase B (Akt) pathways [5]. Further downstream of the ErbB signaling cascade are various transcription factors that are necessary for regulating gene expression. Accordingly, these genes are translated into proteins that comprise the output: apoptosis, cell migration, growth, cell adhesion, and differentiation [5].

The role of the ErbB signaling pathways in normal physiological functions is illustrated in Fig. 1-1 [4]. While dysregulated ErbB signaling pathways are implicated in the enhancement of mitogenic and transforming potential, the ErbB proteins are especially important for organogenesis and differentiation of various cell types, such as Schwann cells and oligodendrocytes [4]. As shown in Fig. 1-1 the ErbB receptors have important physiological roles in the mammary gland, heart, brain, and in Schwann cell development. Section 1.1.3 will provide a more detailed discussion on ErbB proteins—particularly ErbB2—and their role in cardiac development.

1.2 Characterization of ErbB2

The protooncogene *ErbB2/Her2/Neu* encodes the 185 kDa ErbB2 receptor tyrosine kinase that localizes to the plasma membrane, nucleus, and mitochondria[10]. Among the family of four ErbB proteins, ErbB2 is the only receptor that lacks a soluble

ligand. However, ErbB2 is the preferred heterodimerization partner of EGFR, ErbB3, and ErbB4 and is therefore known for its signaling potency as a co-receptor. Heterodimerization with ErbB2 results in autophosphorylation of tyrosine residues on the ErbB2 receptor. The different components of the ErbB2 receptor are illustrated in Fig. 1-2. Of particular importance is the extracellular domain (ECD) that is proteolytically cleaved in ErbB2-overexpressing breast cancers[11-14]. The shedding of the ECD renders the truncated ErbB2 protein to become more active and increases its kinase activity [11].

1.3 Importance of ErbB2 in the heart

The overexpression of ErbB2 has been implicated in several types of cancer such as ovarian and breast due to its enhanced mitogenicity resulting from dysregulated ErbB2 signaling[15-18]. Among all of the cancer types, ErbB2-overexpressing breast cancer is the most widely studied. An estimated 15-20% of invasive ductal breast cancers occur as a consequence of ErbB2 gene amplification that confers a proliferative potential for tumor cells and is correlated with a poor clinical outcome[19, 20].

Initial efforts to characterize the functional and physiological significance of ErbB2 led to the discovery that genetic deletion of ErbB2 is deleterious for neural and cardiac development [21]. The findings from this particular study published in 1995 by K.F. Lee et al. show that neural deficits in ErbB2 null mice were observed as early as E10.5 at the level of the cranial neural-crest-derived neurons, suggesting an important role of ErbB2 in neural development [21]. In addition to the deleterious effects on neurogenesis, the investigators noted that embryos lacking ErbB2 did not survive beyond E11, potentially as a result of impaired cardiac development. Indeed, the mutant embryos failed to form ventricular trabeculae that are necessary for maintaining blood flow during

the early stages of heart development [21]. Interestingly, mice that are deficient in neuregulin display similar cranial and cardiac phenotypes as ErbB2 null mice, while ErbB4 mutant mice, similar to ErbB2, do not survive past E10.5 due to the absence of cardiac trabeculae [22-24]. Subsequent studies using a cardiomyocyte-specific conditional ErbB2 mutant show that ErbB2 mutant mice display impaired cardiac function postnatally that is characteristic of dilated cardiomyopathy[25, 26]. The significance of these findings is that ErbB2 is essential for the normal function of the adult heart and drugs that inhibit ErbB2, such as Trastuzumab/Herceptin, may prevent cardioprotective ErbB2 signaling. Taken together, these studies provide substantial evidence for the crucial role of the ErbB receptors in cardiac function and development [27].

The mechanisms that are responsible for the deleterious effects on the heart from ErbB2 inhibition have been investigated in various *in vitro* and *in vivo* models [28-31] . These studies demonstrate that when ErbB2 is inhibited in the cardiomyocyte, apoptosis occurs through mitochondrial- and reactive oxygen species (ROS)-dependent pathways. For example, neonatal rat ventricular myocytes (NRVM) that are treated with an anti-ErbB2 antibody express more of the pro-apoptotic Bcl-xS protein and have less Bcl-xL protein that promotes cell survival[30]. A shift towards Bcl-xS has also been observed in left ventricular tissues from heart failure patients with a concurrent downregulation of both ErbB2 and ErbB4 mRNA and protein [32]. Inhibition of ErbB2 in NRVM reduces the mitochondrial membrane potential ($\Delta\varphi$) and adenosine triphosphate (ATP) content, suggesting that mitochondrial dysfunction is a consequence of ErbB2 inhibition[28, 30]. Furthermore, increased ROS production is thought to accompany mitochondrial impairments that precede cellular death. Conversely, ErbB2-overexpressing mice have

increased levels of the pro-survival Bcl2 and Bcl-xL proteins and decreased expression of Bcl-xS protein[33]. The cardioprotective role of ErbB2 is supported by additional studies where cardiomyocytes treated with neuregulin-1β (NRG-1β; ErbB4 ligand for which ErbB2 is a co-receptor) have upregulated antioxidant mRNA and proteins [34]. In a separate study, adult rat ventricular cardiomyocytes (AVRM) treated with NRG-1 are protected against doxorubicin-induced oxidant stress and have increased mRNA for glutathione reductase [35]. Thus, multiple models of ErbB2 ablation and overexpression in the heart provide potential mechanistic insight on how pharmacological inhibitors of ErbB2 are detrimental to the heart, as discussed in the following section.

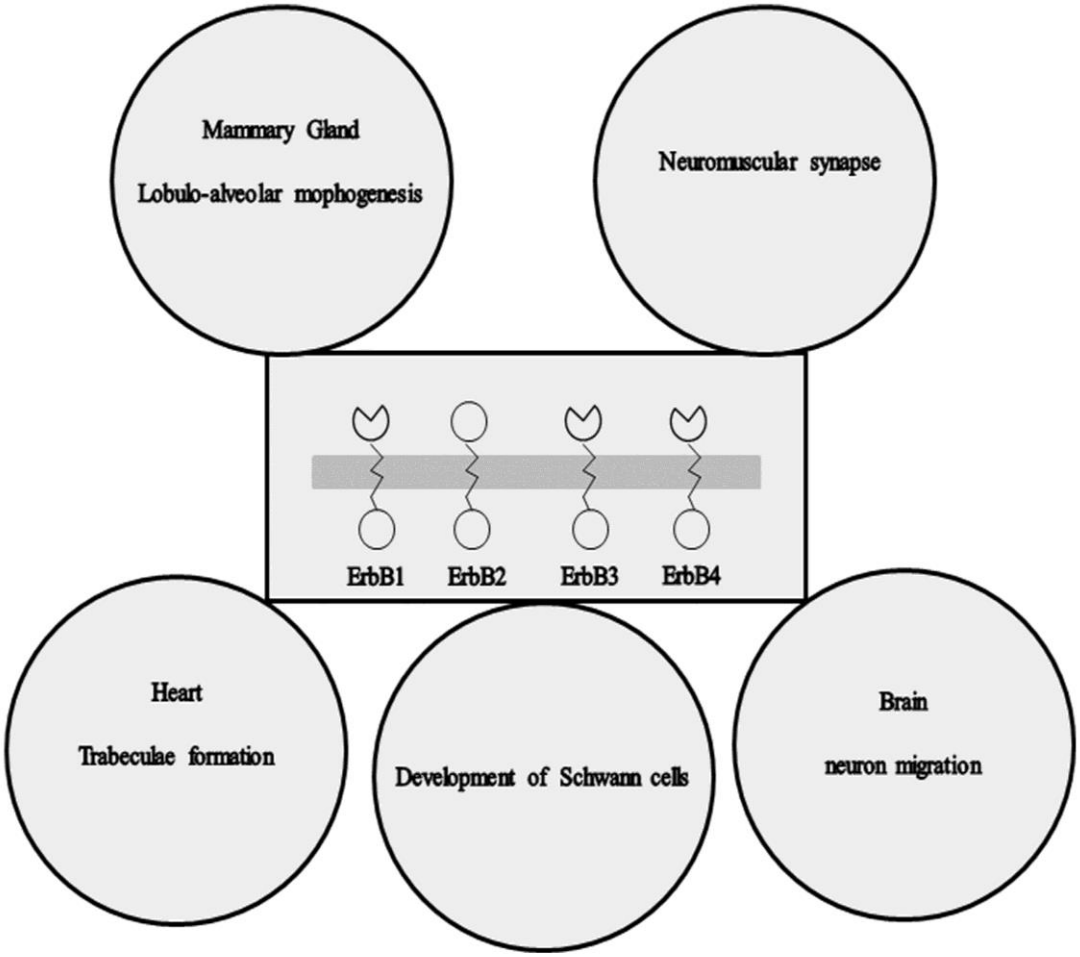


Fig. 1-1. The four ErbB proteins have important physiological functions in organogenesis and cellular differentiation. Coordination between the ErbB receptors and their ligands enable these processes to occur, such as in the development of the brain and heart.

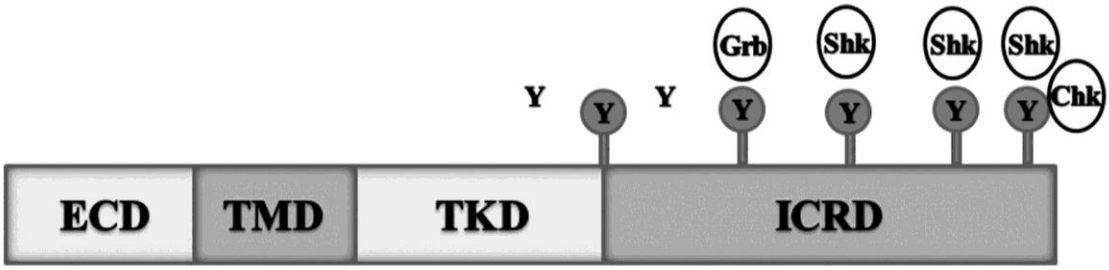


Fig. 1-2. Schematic of the ErbB2 receptor and the various proteins (i.e. SH2-substrates) that can interact with the tyrosine moieties for subsequent activation of signal transduction pathways. ECD, extracellular domain; TMD, transmembrane domain; TKD, tyrosine-kinase domain; ICRD, intracellular regulatory domain; Y, phosphotyrosine residues; Grb, growth factor receptor-bound protein; Shk, SH2 domain-bearing protein kinase; Chk, Csk family tyrosine protein kinase.

1.4 Clinical problem: synergistic cardiac toxicity from doxorubicin and trastuzumab (Herceptin/anti-ErbB2) to treat ErbB2 over-expressing cancers

In 1998, trastuzumab/Herceptin was approved by the Food and Drug Administration (FDA) to treat ErbB2-overexpressing metastatic breast cancer [2]. The efficacy of the drug is attributed to its mechanism of action as a recombinant monoclonal antibody that targets receptor tyrosine kinase signaling. Discovered by researchers at Genentech, trastuzumab is considered the first therapeutic RTK inhibitor that was developed through a genomic-based approach [2]. When administered with the anthracycline doxorubicin to treat ErbB2-overexpressing breast cancer, patients who received the combination therapy experienced an increase in time to both disease progression and treatment failure and an overall improved response compared to either drug alone [36] . Despite the enhanced efficacy from the combination drug therapy, adverse cardiotoxic effects occurred in a subgroup of patients[36]. A retrospective analysis of data from patients involved in the trastuzumab clinical trials reveal a higher incidence of cardiotoxicity and heart failure in patients who received the combination therapy, thus prompting further studies to investigate the underlying mechanism for this cardiotoxicity [36, 37]. Presently, the mechanism for trastuzumab-induced cardiotoxicity is unknown; however, varying explanations for the drug-induced cardiotoxicity include altered cellular structure, oxidative stress, and changes to calcium homeostasis in cardiac myocytes [37]. In a separate study that utilized radiolabeled trastuzumab to identify the uptake of the drug into the myocardium and tumor, the investigators found myocardial uptake in those patients with cardiotoxicity [38]. These studies suggest that individuals expressing cardiac ErbB2 may be susceptible to adverse cardiac outcomes to trastuzumab.

1.5 Doxorubicin induces ErbB2 protein in the heart

Anthracyclines such as doxorubicin are known for their chemotherapeutic value but have the undesirable effect of causing cardiotoxicity. As described in the preceding section, combined treatment of doxorubicin with Herceptin is more efficacious against the treatment of cancer; however, this drug therapy combination may lead to cardiac dysfunction. Although the underlying mechanism for this cardiac toxicity is poorly understood, there is strong support that targeting ErbB2, particularly in the heart, is detrimental to cardiac function and development. Thus, while it has been shown that cardiomyocytes lacking ErbB2 have greater sensitivity to doxorubicin [39], other studies investigated the effects of doxorubicin on ErbB2 expression in the heart [40]. In these studies, rats were administered a specified cumulative dose of doxorubicin over several weeks [40]. Cardiac function of the animals was assessed via echocardiography for changes in cardiac parameters such as contractility and fractional shortening. Doxorubicin treatment reduced both parameters in a dose-response manner; the decrease in contractility was accompanied with an increase in the number of TUNNEL-positive staining cells and nuclei that are indicative of cellular death [40]. Another important finding from the study is that doxorubicin induces ErbB2 protein levels and additional proteins that are activated downstream of the ErbB2 signaling pathway[40]. Taken together, these findings provide a possible mechanism for the combined drug cardiotoxicity of doxorubicin and Herceptin, whereby ErbB2 protein induced by doxorubicin increases the susceptibility of the heart to the ErbB2 inhibitory effects from Herceptin.

Chapter 2

Cardiac-specific overexpression of ErbB2 induces pro-survival pathways and hypertrophic cardiomyopathy in mice

The following chapter includes figures and text that were published in ***PLoS ONE* 7(8): e42805. doi:10.1371/journal.pone.0042805**, and are reproduced here under the terms of the Creative Commons Attribution License.

2.1 Abstract

The essential role of ErbB2 in cardiac function and development is characterized in various studies that describe adverse cardiac effects as one of the major consequences of attenuated ErbB2 signaling. The importance of ErbB2 in the heart is further highlighted in cancer studies in which the incidence of cardiotoxicity is increased when patients with ErbB2-overexpressing breast cancer are treated with a combination drug therapy consisting of doxorubicin and the monoclonal antibody trastuzumab/Herceptin that directly targets ErbB2.

Though numerous studies have explored ErbB2 inhibition in the heart and cardiomyocytes, the consequences of cardiac ErbB2 overexpression are not fully understood. Thus, two lines of transgenic mice were developed that over-express ErbB2 in the heart. The most striking phenotype that the transgenic animals develop is a concentric cardiac hypertrophy with an absence of heart failure and no effect on life span. Compared to wild type mice, the ErbB2 transgenic animals have larger cardiomyocytes, enlarged nuclei, and myocardial disarray. Additional cardiac features of the transgenic mice assessed via echocardiography show similar characteristics as observed in patients with hypertrophic cardiomyopathy (HCM). Furthermore, cardiac ErbB2 overexpression activates signaling proteins that are involved in the ErbB2 pathway, such as EGFR, ErbB3, ErbB4, PI3K subunits, and phosphorylated AKT. The hypertrophic phenotype is further supported by the upregulation of proteins involved in translation, while the shift towards the anti-apoptotic Bcl2 and Bcl-Xl proteins supports the role of ErbB2 in cardioprotection. Lastly, the Erb1/2 inhibitor, lapatinib, reduces the heart weights of the transgenic animals and inactivates the hypertrophic signaling. To our knowledge, this is

the first ErbB2 transgenic model to demonstrate that chronically over-expressed cardiac ErbB2 activates protective signaling pathways and induces hypertrophy that has shared features with HCM models.

2.2 Introduction

As described in section 1.1.1 of the first chapter, the normal physiological role of the ErbB family of RTKs is for cellular differentiation and organogenesis. For example, ErbB2 is critical for cardiac function and development. However, dysregulated ErbB2 signaling is implicated in cancer for its mitogenic potential. In the case of ErbB2-overexpressing breast cancer, a poor clinical outcome is often a consequence of the enhanced proliferative capacity of the tumor cells. Drugs such as Herceptin are effective for treating ErbB2-overexpressing breast cancer and its efficacy increases when it is combined with the anthracycline doxorubicin. However, as previously discussed in section 1.2 the incidence of cardiotoxicity increases when both drugs are administered, prompting further studies on the adverse effects from this cancer treatment regimen. In order to investigate the role of uninhibited ErbB2 *in vivo*, we generated two lines of transgenic mice with cardiac-specific ErbB2 overexpression. The transgenic construct includes the alpha-myosin heavy chain (αMHC) located in the promoter region that confers cardiac-specific ErbB2 overexpression. Two founder mice were identified via Southern Blotting to contain the ErbB2 transgene; these animals were subsequently used to generate two separate lines of ErbB2 transgenic breeding colonies.

The most striking phenotype of the ErbB2 transgenic mice is a markedly enlarged heart, as evidenced by hematoxylin-eosin (H&E) stained transverse cross sections and an enlargement of the atria and ventricles compared to the wild type hearts (data not shown).

M-mode echocardiography confirmed the presence of concentric hypertrophy, whereby the heart walls are hypertrophied and the left ventricular chamber is reduced in the transgenic hearts. Additional echocardiographic studies (e.g. left ventricle wall thickness and mass) to assess cardiac function confirm the concentric hypertrophy phenotype.

A possible consequence of cardiac hypertrophy is cardiac dysfunction that can lead to heart failure. Despite the observed hypertrophic phenotype, there is an absence of heart failure in the transgenic animals. For example, the fractional shortening, which is a measure of left ventricle systolic function (represented by the following equation: (LVEDd-LVESd)/LVEDd)) x100%), is within the range of normal cardiac function. Further support for the lack of heart failure is seen in immunoblots that show a shift towards the anti-apoptotic, pro-survival Bcl-xL and Bcl-2 proteins (data not shown). As discussed in section 1.1.3, the levels of pro-apoptotic proteins such as Bcl-xS are known to increase during heart failure and when ErbB2 is inhibited.

Additional studies with the ErbB2 inhibitor lapatinib provide additional evidence for ErbB2 and its induction of hypertrophy. In these studies, mice at P4.5 (postnatal day 4.5) and P10.5 were administered lapatinib via oral gavage. In both cases, the heart weights of the transgenic animals were reduced and less ErbB2 protein was expressed with lapatinib treatment (data not shown). Thus, the lapatinib studies support the role of ErbB2 in cardiac hypertrophy and show that hypertrophy was reversed in younger and older animals.

2.3 Results and Discussion

The difference in heart weight between the wild type and transgenic mice was detected as early as P9.5 (Fig. 2-1) and the difference remains when heart weight is

normalized to body weight (Fig. 2-2). These results suggest that the hypertrophic phenotype from overexpressed cardiac ErbB2 may begin after P6.5 and can be reversed with lapatinib treatment as shown in Fig. 2-1 and 2-2. Subsequent studies to further characterize the ErbB2 transgenic mouse model confirm the presence of concentric hypertrophy with features that are consistent with those observed in HCM patients. These features include cardiomyocyte hypertrophy, myocardial disarray, and interstitial fibrosis. Thus, our model of cardiac ErbB2 overexpression can be a potentially used as a model for human HCM.

The most significant finding from the cardiac ErbB2 overexpression animal model is the distinctive concentric hypertrophic phenotype that does not progress to heart failure. Furthermore, signaling pathways that are known to be involved downstream of ErbB2, particularly in cancer etiology, are activated such as proteins that regulate translation and anti-apoptotic processes that potentially mediate the occurrence of hypertrophy. To our knowledge, the ErbB2 transgenic model provides the first evidence that ErbB2 overexpression induces the protein expression of the remaining three ErbB members and the PI3K-110 and -85 subunits. Our transgenic model provides further insight on the functional importance of ErbB2 in the heart, as the mechanisms that underlie cardiac ErbB2 induction are currently unknown. Conversely, the studies on the implications for ablated and inhibited cardiac ErbB2 highlight the crucial role of ErbB2 in cardiac function and development. Thus, our ErbB2 transgenic model provides new insight on ErbB2 and cardiac hypertrophy that is potentially useful for understanding the functional significance of ErbB2 protein in the human heart.

Overexpression of cardiac ErbB2 leads to additional cardiac features that we observed through histological staining and electrophysiological studies. For example, Masson's Trichrome stained heart tissue revealed the presence of an age-related interstitial fibrosis in the ErbB2 transgenic mice. Heart sections stained with hematoxylin and eosin (H&E) show that the ErbB2 transgenic mice have myocardial disarray that is a common morphological feature of HCM. Transgenic mice that were treated with isoproterenol, a non-specific beta-adrenergic agonist, respond similarly as other HCM animal models. Additional electrophysiological studies indicate that the ErbB2 transgenic mice have shared features with HCM patients that include an increased sensitivity to adrenergic stimulation and arrhythmias.

The role of ErbB2 in cardiac hypertrophy was further investigated in studies with the EGFR/ErbB2 kinase inhibitor lapatinib. Transgenic mice that were administered lapatinib had reduced heart weight/body weights and lowered expression of proteins involved in translation. These studies may be useful to identify the underlying mechanisms of hypertrophy and additional mechanisms that are relevant to ErbB2 signaling.

In summary, the ErbB2 transgenic model may further our understanding of why a subgroup of patients with ErbB2-over-expressing breast cancer are more vulnerable to cardiac toxicity from the cancer therapies Herceptin and doxorubicin. It is possible that in patients with varying degrees of cardiac hypertrophy where ErbB2 is involved, inhibiting ErbB2 activity (such as in cancer therapy) may lead to heart failure. Therefore, the ErbB2 transgenic model can be used to explore other research avenues for the critical role of ErbB2 in the heart.

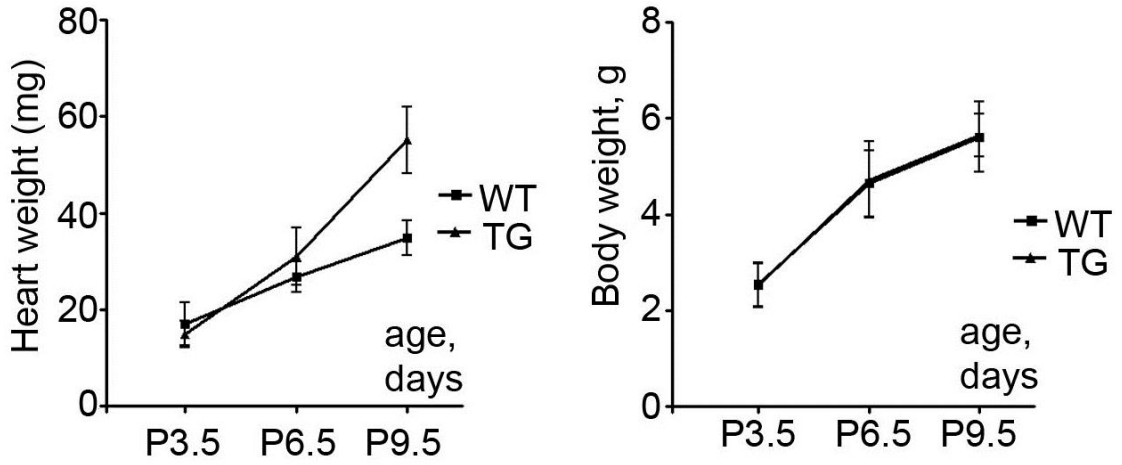


Fig. 2-1. Heart and body weights of wild type (WT) and ErbB2 transgenic (TG) mice at various postnatal (P) ages. The heart weights of the TG mice begin to increase after P6.5 compared to WT hearts.

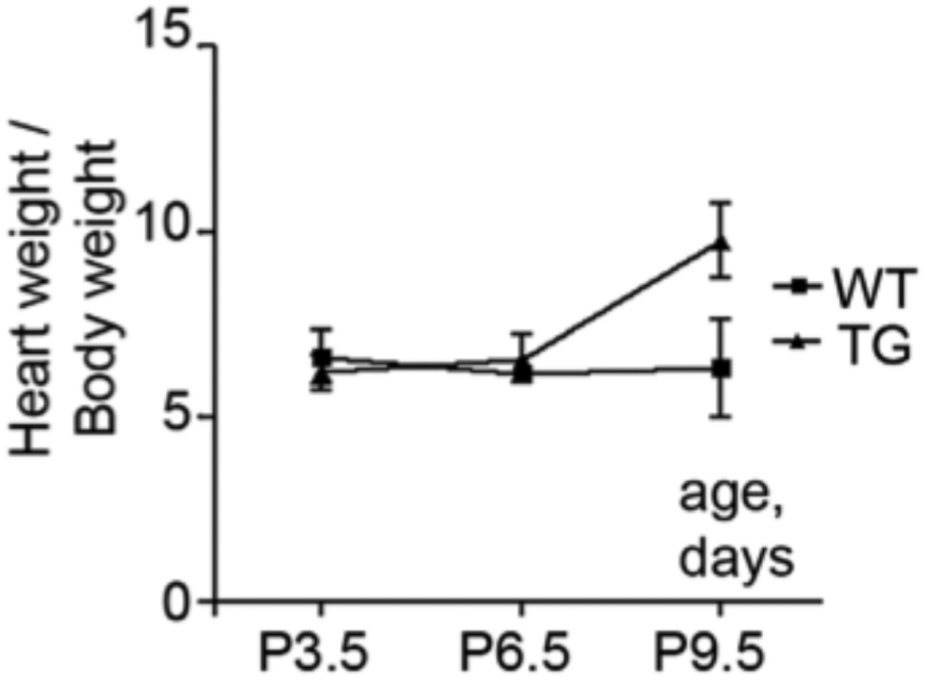


Fig. 2-2. Heart weight normalized to body weight of the WT and TG hearts at various postnatal ages. The ratios of heart weight to body weight indicate that the hypertrophy in TG mice begins after P6.5.

2.4 Methods and Materials

Animals

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Johns Hopkins Medical Institutions (Animal Welfare Assurance # A-3273-01).

Measurement of neonatal heart and body weights

Wild type and ErbB2 transgenic mice were euthanized and weighed at P3.5, 6.5, and 9.5 (postnatal days) through decapitation. The hearts were excised and weighed; hearts were snap frozen and saved for additional studies. The heart weight/body weight was subsequently determined for all three age groups.

Statistical Methods

GraphPad Prism software (GraphPad, La Jolla, CA) was used to perform statistical analysis. After determining means and standard deviations, the Student unpaired T-test was used to determine significance of differences between groups, with a p-value of <0.05 accepted as a significant difference.

Chapter 3

Overexpression of Epidermal Growth Factor Receptor 2 (ErbB2) in the Heart Alters Redox-Sensitive Molecules that Attenuate Oxidant Stress

3.1 Abstract

Cardiac-specific overexpression of epidermal growth factor receptor 2 (ErbB2) in mice activates signaling pathways that induce hypertrophy without progression to heart failure. However, the transition from hypertrophy to heart failure is mediated by oxidative stress. Furthermore, in other models of cardiac hypertrophy with an absence of heart failure, glutathione peroxidase (GPx) is activated as a cardioprotective response. In order to further understand the lack of heart failure protective phenomenon, we investigated whether ErbB2 transgenic (ErbB2^{tg}) mice have increased antioxidant protection against reactive oxygen species (ROS). Our results indicate that there is less ROS in the hearts and cardiac mitochondria from the ErbB2^{tg} mice. We hypothesized that the cardiac protection afforded in the ErbB2^{tg} mice is due to the Abelson [41] nonreceptor tyrosine kinases (nRTKs) c-Abl and Arg (Abelson-related gene) that in cancer studies are downstream of ErbB2 and in other studies are known to activate GPx. Both nRTK proteins were upregulated in the ErbB2^{tg} hearts and co-immunoprecipitation assays show that c-Abl and Arg form a complex with ErbB2 protein. To our knowledge, these are the first studies to demonstrate ErbB2-Abl kinase protein interaction in the heart. ErbB2^{tg} mice have significantly increased levels of glutathione peroxidases (GPx) 1, 3, and 4 proteins and elevated GPx activity. Treatment with lapatinib (ErbB2/EGFR kinase inhibitor) reduced c-Abl and Arg protein expression and subsequent GPx activity. We established an *in vitro* model of ErbB2 overexpression and performed a siRNA-mediated targeted knockdown of ErbB2 using H9c2 cells to further investigate an ErbB2-dependent mechanism for protection against oxidative stress and cellular death, particularly against doxorubicin. Overexpression of ErbB2 in H9c2 cells results in

increased protection against doxorubicin-induced oxidative stress and cellular death; abrogation of ErbB2 reduces the protective effects against doxorubicin. GPx activity was found to be independent of ErbB2 signaling in the H9c2 model of ErbB2 overexpression and knockdown. However, preliminary studies with neonatal mouse cardiomyocytes revealed that GPx activity is higher in the ErbB2^{tg} mouse hearts, suggesting a requirement for the hypertrophic phenotype and that the neonatal cardiomyocytes are the more appropriate *in vitro* model four our studies compared to the H9c2 cells. Our results demonstrate that the hypertrophic phenotype activates GPx to reduce ROS as a potential cardioprotective mechanism to prevent the progression from hypertrophy to heart failure in the ErbB2^{tg} mice. We propose a novel multi-protein connection whereby ErbB2/EGFR drives GPx activation through c-Abl and Arg to attenuate oxidant stress. Thus, cardiac ErbB2 is essential for maintaining antioxidant defenses that protect against oxidative damage and heart failure. Lastly, anti-ErbB2 and anti-nRTK drugs may adversely affect the redox signaling pathways that are necessary for maintaining anti-oxidant defenses in cardiac hypertrophy and potentially in other cells that are dependent on these pathways.

3.2 Introduction

In the previous chapter, the phenotypic changes that resulted from the selective overexpression of ErbB2 in the cardiomyocyte were described. Among the changes that occurred as a result of chronically overexpressed ErbB2 is a distinctive cardiac hypertrophy that does not progress to heart failure. The ErbB2 transgenic murine model is particularly useful for cardiac studies as ErbB2 knockout mice die prematurely, thus precluding the study of specific mechanisms of cardiac protection. The lack of heart failure phenotype in the ErbB2^{tg} mice led us to hypothesize that ErbB2 may induce the

up-regulation of protective antioxidant enzymes, as previously seen *in vitro* with cardiomyocytes that were treated with neuregulin-1β (NRG-1), a ligand for ErbB4 and a hetero-dimerization partner of ErbB2 [34, 35]. Most importantly, however, is that the transition from hypertrophy to heart failure is mediated by oxidative stress [42, 43], prompting us to further investigate whether ErbB2 overexpression enhances the antioxidant capacity in the heart to subsequently reduce oxidant stress.

Several studies have shown that glutathione peroxidase (GPx) activity is elevated in chronic cardiac hypertrophy and is reduced in heart failure [44-46]. Additionally, there is evidence to suggest that EGFR (ErbB receptor that is up-regulated by ErbB2 in the transgenic mice [33]) mediates GPx activity in order to reduce ROS levels [47]; however, the role of ErbB2 was not investigated. Support for the possible involvement of GPx in the ErbB2^{tg} mice is based on microarray data in which the GPx1 and GPx3 genes are upregulated as indicated by the z ratios (calculated from the z scores for individual genes shown in Fig. 3-2 [48]) for oxidative stress genes shown in Fig. 3-1. We performed quantitative real-time polymerase chain reaction (qRT-PCR) with cDNA from the hearts of ErbB2^{tg} and wild type mice to validate the microarray data (Fig. 3-3). Although the differences in mRNA were not statistically significant for either gene, the data provided a rationale for us to further explore a potential protective role of GPx in the hearts of the ErbB2^{tg} mice.

In order to investigate the mechanism for the protective effects of ErbB2 overexpression, particularly against ROS and cellular death, we over-expressed ErbB2 *in vitro* and genetically knocked down ErbB2 via siRNA in H9c2 rat cardiomyoblast cells. Preliminary experiments using neonatal cardiomyocytes that were isolated from wild type

and ErbB2^{tg} hearts allowed us to examine whether the hypertrophic phenotype is necessary to regulate GPx activity in the ErbB2^{tg} mice. Thus, these *in vitro* models were employed to facilitate our understanding of a potential antioxidant-signaling pathway that is unique to cardiac ErbB2 overexpression *in vivo* and in the setting of hypertrophy.

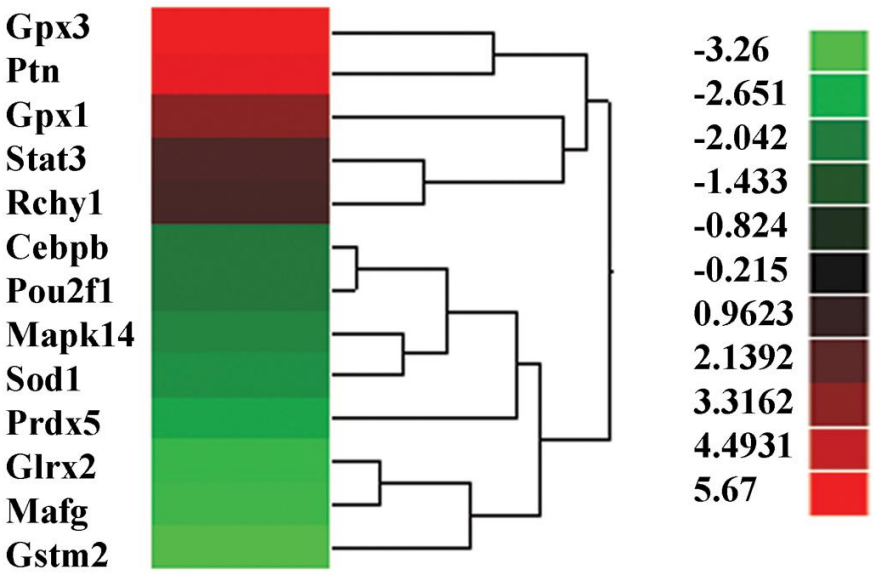


Fig. 3-1. **Microarray data of oxidative stress genes suggest that ErbB2 overexpression results in the upregulation of glutathione peroxidases 1 and 3 (GPx1 and GPx3).** A heat map of oxidative stress genes sorted from the microarray data that were based on the Z ratio that was calculated from the Z scores (n=3 per group). Scale of red to green represents higher to lower gene expression.

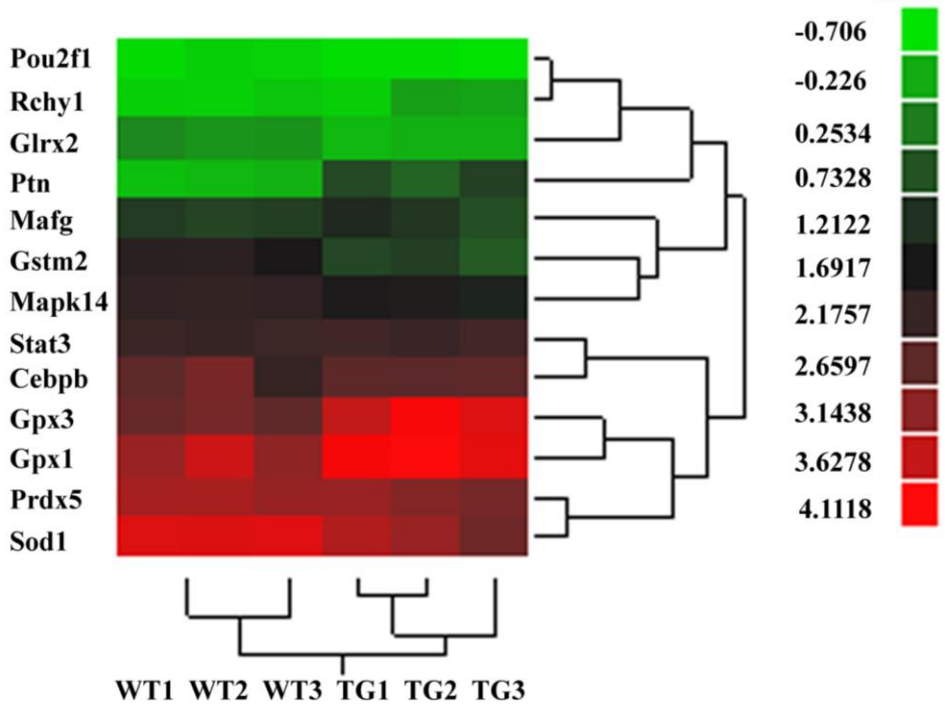


Fig. 3-2. Z scores obtained from individual samples. The Z scores displayed in the heat map were used to calculate the Z ratio of the individual oxidative stress genes (n=3 per group). Scale of red to green represents higher to lower gene expression.

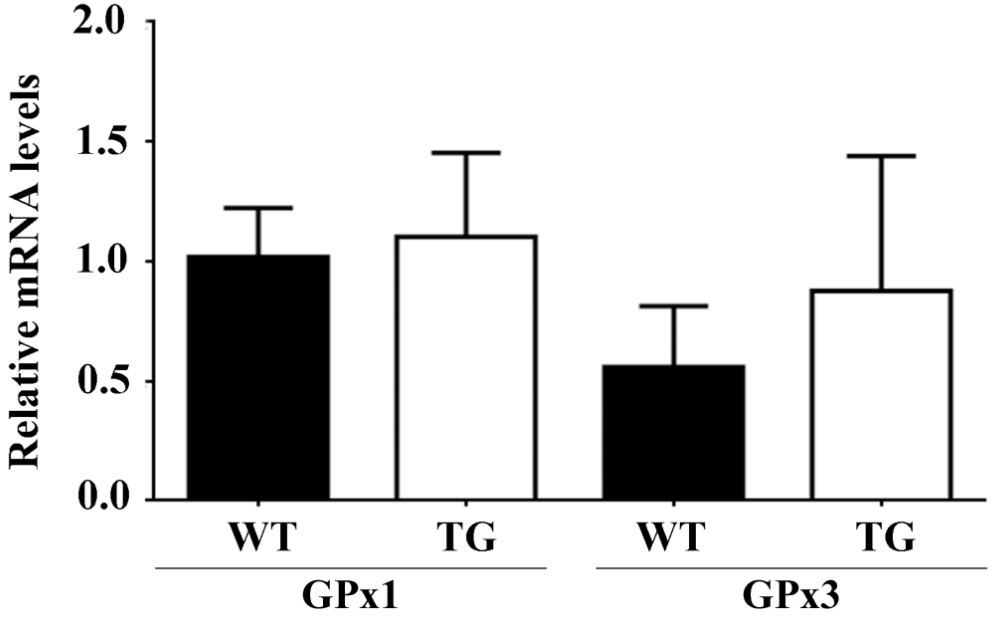


Fig. 3-3. Validation of GPx1 and GPx3 from the microarray data. Quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis of Gpx1 and Gpx3 do not show any differences in mRNA expression (n=8 per group). Values are means ± SD.

3.3 Results

3.1 ErbB2^{tg} hearts have lower levels of ROS

In vitro studies have shown that ErbB2 pathway activation in adult rat cardiomyocytes treated with neuregulin-1β causes an up-regulation of antioxidants [49] and protection against oxidative stress [35]. Results from analyses that utilized the oxidative stress fluorescent indicator 2,7-dichlorodihydrofluorescein diacetate (DCF-DA) and electron paramagnetic resonance (EPR) with left ventricular homogenates (Fig. 3-4 A, B) show that the ErbB2^{tg} hearts produce less oxidative stress and superoxide (O_2^-), respectively, compared to WT hearts. Since the mitochondria are important producers of ROS [50-52], we measured hydrogen peroxide (H_2O_2) production in mitochondria using an Amplex Red assay. Isolated heart mitochondria from the ErbB2^{tg} hearts release less H_2O_2 than WT heart mitochondria (Fig. 3-4 C). Further experiments to assess protein carbonylation as a marker of oxidative damage in whole heart lysates did not show any differences between both groups of animals (Fig. 3-4 D).

3.2 ErbB2 overexpression up-regulates nonreceptor tyrosine kinases (nRTKs) c-Abl and Arg that are important in glutathione peroxidase activation

A possible mechanism for ErbB2 signaling that leads to GPx activation may be through the nRTKs c-Abl and Arg. First, i*n vitro* studies have shown that GPx activity is regulated by c-Abl and Arg [53]. Secondly, in separate studies ErbB2 is known to activate and bind to c-Abl and Arg in cancer cells, thus providing further support for the potential role of these nRTKs as signaling intermediates for the downstream activation of GPx in the ErbB2^{tg} hearts [54, 55]. To our knowledge, there are no studies that link ErbB2 to the up-regulation of nRTK with subsequent GPx activation. Immunoblots and densitometry

analysis of phosphorylated and total c-Abl indicate that the transgenic hearts express more of these proteins than the wild type hearts (Fig. 3-5 A). Phosphorylation at the tyrosine 245 moiety located in the kinase domain of c-Abl contributes to c-Abl kinase activation[56]. Immunoprecipitation results show that c-Abl binds to ErbB2 in the transgenic hearts; the interaction between c-Abl and ErbB2 has been shown previously in cancer cells [54, 55] but not in the heart (Fig. 3-5 B). Our previous studies on ErbB2 overexpression in the heart showed a concurrent up-regulation of EGFR [33], an ErbB family receptor that is increased when ErbB2 is elevated in cancer cells [57, 58]. Furthermore, EGFR binds to and activates Abl kinases in breast cancer cells [55]. Separate *in vitro* studies have shown that activated Abl kinases phosphorylate EGFR and prevent ligand-dependent internalization of the receptor[59]. Our immunoblotting results show that c-Abl binds to EGFR as well as ErbB2 (Fig. 3-5 B). An additional nRTK, Arg, is also elevated under the control of ErbB2 in the heart (Fig. 3-5 C) and an immunoblot from an immunoprecipitation assay shows that Arg protein also binds to ErbB2 protein in the transgenic hearts (Fig. 3-5 D). Total GPx activity in whole heart lysates and in isolated heart mitochondria from WT and ErbB2^{tg} mice were measured and GPx activity was increased in the ErbB2^{tg} hearts (Fig. 3-5 E, F). The redox- cycling of GPx to remove H_2O_2 requires glutathione (GSH). Our results indicate that the levels of GSH were not different between the two groups of animals (Fig. 3-5 G). Since glutathione peroxidases can exert cardioprotection [60-62], this enzyme may be involved in pro-survival pathways *in vivo* under the control of ErbB2 protein expression [33]. Glutathione peroxidases (GPx) 1, 3, and 4, in particular, are known to exert antioxidant cardioprotective effects [63] and have been shown to be important in cardiac hypertrophy [64, 65]. Immunoblotting results (Fig. 3-5

H) to assess GPx1 protein expression indicate that the ErbB2^{tg} hearts express GPx1 at a significantly higher level than WT hearts. Cytosolic and mitochondrial fractions prepared from the hearts of both WT and ErbB2^{tg} animals were probed for GPx1 and immunoblots (Fig. 3-5 I) show that GPx1 protein is highly expressed in both the cytoplasm and mitochondria of the ErbB2^{tg} hearts compared to the WT hearts. The protein levels of two additional glutathione peroxidases, the extracellular GPx3 and GPx4, a phospholipid hydroperoxidase, are also elevated in ErbB2^{tg} hearts compared to WT hearts (Fig. 3-5 J, K).

3.3 Lapatinib treatment reduces the expression of the Abl kinases and GPx activity.

In order to determine if ErbB2/EGFR kinase signaling is important for the activation of glutathione peroxidases via c-Abl and Arg, eight- to ten-week-old male mice were treated with the ErbB2/EGFR inhibitor lapatinib orally for 11 days. Treatment with lapatinib reduces both c-Abl and Arg protein expression in ErbB2^{tg} whole heart homogenates (Fig. 3-6 A, B). This nRTK reduction was accompanied by a concurrent decrease in GPx3 protein and a significant decrease GPx enzyme activity under lapatinib treatment (Fig. 3-6 C, E).

3.4 Overexpression of ErbB2 in the heart increases the levels of p-Src (Tyr416) protein

A possible signaling intermediate that is downstream of ErbB2 is the non-receptor tyrosine kinase Src that has been shown to interact with ErbB2 in breast cancer and activates Abl kinases[41, 66]. The tyrosine 877 (Tyr877) moiety is located in the kinase domain of ErbB2 and is homologous to the tyrosine 416 (Tyr416) moiety in the activation loop of the Src kinase domain[67]. Furthermore, phosphorylation at Tyr416 is required for Src enzymatic activity[67].

As expected, the ErbB2^{tg} hearts have higher levels of ErbB2 protein that is phosphorylated at Tyr877, rendering an active ErbB2 protein (Fig. 3-7 A). Additionally, immunoblots show that the ErbB2^{tg} mouse hearts have an upregulation of p-Src (Tyr416), suggesting that Src is in its active form and can subsequently activate the Abl kinases (Fig. 3-7 B).

3.5 Glutathione reductase and thioredoxin reductase activity and protein levels are not different in the ErbB2^{tg} mouse hearts

Since it has been shown that neuregulin-1β treatment up- regulates glutathione reductase (GR) mRNA in adult rat ventricular cardiomyocytes [35], we measured GR protein expression and activity. The results from the immunoblot and activity assay (Fig. 3-8 A, C) indicate that there is no difference in GR protein expression or activity between WT and ErbB2^{tg} mice. Next, we assessed whether the thioredoxin pathway is involved in the redox signaling in ErbB2^{tg} hearts. Thioredoxin reductase 2 (TrxR2) protein and activity were measured in whole heart homogenates and mitochondria, respectively, and neither was significantly changed in WT and ErbB2^{tg} mice (Fig. 3-8 B, D).

3.6 H9c2 cells over-expressing ErbB2 are protected against doxorubicin-induced oxidative stress and cellular death

Transient overexpression of ErbB2 in the H9c2 rat cardiomyoblast cell line results in a greater than two-fold difference in ErbB2 protein levels compared to the vector control (Fig. 3-9 A) and an immunoprecipitation assay indicates that ErbB2 is phosphorylated (Fig. 3-9 B). Additionally, ErbB2 overexpression concurrently upregulates EGFR protein (Fig. 3-9 C). A widely known consequence for inhibiting ErbB2 in the heart is cellular death [28-31]. While many studies have shown the deleterious

effects from blocking ErbB2, the potential protective effects from ErbB2 overexpression, to our knowledge, have not been reported. Our results indicate that ErbB2 is protective against oxidative stress from doxorubicin treatment as measured by the DCF-DA fluorescent assay (Fig. 3-9 D). H9c2 cells that over-express ErbB2 have more active mitochondrial dehydrogenase as assessed by the MTT assay compared to H9c2 cells that were transfected with vector (Fig. 3-9 E).

3.7 siRNA-mediated knockdown of ErbB2 in H9c2 cells reduces the protective effects of ErbB2 against oxidative stress and cellular death from doxorubicin

Since we showed that ErbB2 overexpression in H9c2 cells is protective against doxorubicin-induced oxidative stress and cellular death, we performed a siRNA-mediated knockdown of ErbB2 to assess whether the protective effects against doxorubicin would become reduced. Our results indicate that transfection with ErbB2 siRNA decreases the levels of ErbB2 and EGFR proteins (Fig. 3-10 A, B). Treatment with doxorubicin increases ROS levels when siRNA was used against ErbB2 at the highest dose of doxorubicin (Fig. 3-10 C). The protective effect against doxorubicin was reduced only at the 0.5 μM dose of doxorubicin, suggesting that protection against doxorubicin-induced cellular death is not entirely dependent upon ErbB2 at higher doses of doxorubicin (Fig. 3-10 D). Alternatively, genetically ablated ErbB2 may induce ErbB2 protein upon exposure to doxorubicin, as rats treated with doxorubicin had an upregulation of ErbB2 protein in their hearts[40].

3.8 Overexpression and knockdown of ErbB2 in H9c2 cells does not alter GPx activity

As demonstrated by our *in vivo* findings, GPx protein and activity are increased in the ErbB2^{tg} mice. In order to more closely examine whether ErbB2 regulates GPx

activity we performed GPx enzymatic assays using H9c2 cells under the conditions of ErbB2 overexpression and knockdown. Our results indicate that GPx activity is independent of ErbB2 signaling as no change in activity was detected in either group (Fig. 3-11 A, B). We proposed that the increase in GPx activity in the ErbB2^{tg} hearts occurred as a result of the hypertrophy induced by ErbB2 overexpression. Preliminary data show that neonatal cardiomyocytes isolated from the ErbB2^{tg} hearts have higher GPx activity compared to WT cardiomyocytes (Fig. 3-11 C). Our results suggest that the hypertrophic phenotype is required for regulating GPx activity in the ErbB2^{tg} mice.

3.4 Discussion

In our transgenic model of cardiac ErbB2 overexpression the mice develop a distinctive hypertrophy that does not progress to heart failure, which is a process that is mediated by oxidative stress. The unique phenotype of the ErbB2^{tg} mice prompted us to investigate whether ErbB2 upregulation in the heart induces antioxidant defenses against ROS. Numerous studies have reported that ErbB2 is essential for protecting against cellular death, oxidative stress, and mitochondrial dysfunction[28-31]; however, the purpose of these studies was to address the deleterious effects from ErbB2 inhibition. ErbB2 has been connected to antioxidant defense mechanisms in cell culture systems where neuregulin-1β (a ligand for ErbB4) treatment upregulates antioxidant enzymes[49]. This signaling likely occurs through either neuregulin-1β-induced hetero-dimerization of ErbB2 and ErbB4 or homo-dimerization of ErbB4[5]. The downstream signaling outcomes from these receptor pairings—either heterodimers or homodimers—require further study but outcomes are likely different[5]. For example, the receptors themselves when over-expressed in mice have different phenotypes. Mice over-expressing ErbB4 do not have a

hypertrophic phenotype[68, 69] whereas ErbB2 over-expressing mice have concentric hypertrophy that does not progress to heart failure[33]. Thus, the receptors may exert distinct control over the signaling to produce hypertrophy outcomes. Neuregulin-induced signaling is likely to vary from ErbB2/EGFR collaboration as we demonstrate with our findings.

We employed three different methods to assess ROS and determined that under increased ErbB2 expression, there are lowered levels of ROS in transgenic hearts and from isolated transgenic heart mitochondria. Since several studies have shown that GPx activity is elevated in chronic cardiac hypertrophy and is reduced in heart failure,[44-46] we investigated the role of ErbB2 in regulating GPx through c-Abl and Arg, nRTKs that are known to be regulated by constitutively active EGFR and ErbB2 in breast cancer cell lines[55]. Additionally, *in vitro* studies have shown that ErbB2 binds to the SH2 domains of Abl kinases as well as phosphorylates c-Abl[54]. We found that ErbB2 and EGFR are associated with c-Abl and that ErbB2 protein binds to Arg in the heart by co-immunoprecipitation assays. Cardiac ErbB2 overexpression significantly increased levels of GPx1, GPx3, and GPx4 proteins, accompanied by an increase in GPx enzymatic activity. Next, we found that the ErbB2/EGFR inhibitor lapatinib reduced protein levels of both c-Abl and Arg followed by a decrease in the levels of GPx3 protein and GPx enzymatic activity, thus supporting the role of ErbB2/EGFR in nRTK pathway activation of GPx in the heart. The modest reduction in GPx activity and lack of change in the expression of GPx1 and GPx4 proteins with lapatinib treatment may be attributed to the length of time that the drug was administered. It is possible that a prolonged treatment with lapatinib that exceeds 11 days, as was done for our studies, will reduce all GPx

proteins and activity even further. Although ErbB2 has been connected to the activation of Src, c-Abl, and Arg in cancer cells [54, 55, 70], and these nRTKs, particularly c-Abl and Arg, have been connected to the activation of GPx in various cell lines [53], we are the first to show the multiple protein connection between ErbB2/EGFR, Src, c-Abl/Arg, and GPx (Fig. 3-12).

The role of ErbB2 on initiating EGFR up-regulation was demonstrated in our previous studies[33] and in multiple cancer models[71]. The heterodimeric pairing of ErbB2 and EGFR, for example, prevents EGFR internalization[5, 72]. Furthermore, Abl kinases regulate EGFR receptor internalization[59], thus providing additional support for a role of Abl kinases in ErbB signaling pathways. While there is evidence for the proximal aspect of our proposed mechanism (i.e. ErbB2 to the Abl kinases) and for the distal signaling pathway (i.e. Abl kinases to GPx), it is possible that EGFR is similarly involved as ErbB2 in signaling to Gpx through the Abl kinases. Therefore, a possible limitation of our study is that the individual contribution from ErbB2 and EGFR to regulate GPx activity through the Abl kinases is not easily distinguishable.

A possible signaling intermediate that is more immediately downstream of ErbB2 that activates Abl kinases is the non-receptor tyrosine kinase Src, which is known to interact with ErbB2 in various breast cancer cell lines[41, 66] and is upregulated in the hearts of the ErbB2^{tg} mice. Src activity is required for the phosphorylation of ErbB2 at the tyrosine 877 (Tyr877) moiety[67]. We found that ErbB2 is phosphorylated at Tyr877, suggesting that Src is active in the ErbB2^{tg} hearts. Furthermore, the interaction between Src and phosphorylated ErbB2 enhances Src activity[67]. In separate studies we have shown through co-immunoprecipitation assays that ErbB2 and Src proteins are bound

together in a complex in the ErbB2^{tg} hearts. Active Src in the ErbB2^{tg} hearts, therefore, is likely to activate the Abl kinases. To our knowledge, ErbB2 signaling to Src and subsequent activation of Abl kinases has not been investigated. Evidence exists, however, for activated c-Abl via growth factor stimulation from the plate-derived growth factor (PDGF) that is upstream of Src[73]. Taken together, our results provide support for a potential mechanism for ErbB2 signaling to Src that is necessary for Abl kinase activation to regulate downstream factors such as glutathione peroxidase[74]. Additional studies are needed to fully characterize this mechanism and to determine whether ErbB2 is regulating Src and Abl kinases in the heart. For future studies, Src inhibitors may be administered to the ErbB2^{tg} mice to assess whether Abl kinase and GPx activity are concurrently downregulated in the ErbB2^{tg} hearts.

In order to strengthen our proposal that ErbB2 is protective against ROS and regulates GPx we established *in vitro* models of ErbB2 overexpression and abrogation using the H9c2 rat cardiomyoblast cell line. Under both conditions, the cells were treated with doxorubicin[75] and ROS production and cellular viability were assessed by DCF-DA and MTT assays, respectively. Our results show that ErbB2 overexpression is more protective against doxorubicin-induced oxidative stress and cellular death compared to vector transfected cells. Genetic knockdown of ErbB2 with siRNA reversed the protective effects from ErbB2 against doxorubicin, with increased ROS levels at the highest dosage of doxorubicin and a decrease in cellular viability at a lower dosage of doxorubicin. Our findings that the protective effects from ErbB2 overexpression against doxorubicin are more robust than the changes we observed from ErbB2 knockdown suggest that compensatory mechanisms are upregulated when ErbB2 is abrogated. For

example, as discussed in Chapter 1, treatment with doxorubicin induces ErbB2 protein in rat hearts. Therefore, it is plausible that despite the removal of ErbB2 via siRNA, doxorubicin is able to effectively upregulate ErbB2 protein so that the level of ROS production and cellular death is close to the levels that were seen in control cells treated with doxorubicin. Furthermore, if ErbB2 protein is upregulated by doxorubicin in the H9c2 cells as a protective mechanism against oxidative stress and cellular death, then ErbB2 overexpression would lead to an additive effect, which we observed in our ErbB2 overexpression assays.

Since the ErbB2^{tg} mice have elevated GPx proteins and activity we employed the *in vitro* models to address whether GPx is regulated by ErbB2 signaling. We determined that ErbB2 overexpression and knockdown in H9c2 cells does not alter GPx enzymatic activity. Though we can conclude from our findings that GPx activation is independent of ErbB2, we cannot immediately rule out the possibility that the hypertrophic phenotype is necessary for GPx activity in the ErbB2^{tg} mouse model. As previously discussed, GPx activity was increased in models of chronic, stable hypertrophy without heart failure[44-46]. Thus, a limitation of the H9c2 *in vitro* model is that it cannot fully recapitulate the phenotypes that occur *in vivo* and in primary cardiomyocytes. While it has been shown that H9c2 cells can be stimulated with endothelin-1 and angiotensin II to acquire hypertrophic properties [76], we chose to utilize primary neonatal cardiomyocytes that were isolated from wild type and ErbB2^{tg} hearts. Compared to H9c2 cells that have the capacity to proliferate, primary neonatal cardiomyocytes are a more relevant *in vitro* model for our studies and can undergo hypertrophy autonomously[77, 78]. Our preliminary studies using pooled neonatal cardiomyocytes from wild type and ErbB2^{tg} hearts indicate

that GPx activity is increased in the ErbB2^{tg} cardiomyocytes compared to wild type myocytes. Until we confirm our findings with additional animals, our results appear promising that the protective antioxidant responses in the ErbB2^{tg} hearts are potentiated by ErbB2 signaling and the associated hypertrophy. Thus, further studies are necessary to determine whether the distinctive up-regulation and activation of GPx by ErbB2-induced signaling may be specifically important in a hypertrophic cardiomyocyte.

Though we did not observe differences in GPx activity in the H9c2 *in vitro* models, the question remains regarding the protective effects from ErbB2 overexpression that are independent of GPx activity. Alternatively, ErbB2 overexpression in H9c2 cells may be due to reduced levels of ROS. Possible clues for alternate sources of antioxidant protection are from studies where treatment of cardiomyocytes with neuregulin-1β induced an increase in gene expression of hypertrophic markers, oxidative stress defenses, and pro-survival pathways, similar to the findings of our current study and previous studies [33, 49]. While other groups found that neuregulin-1β treatment of cardiomyocytes increased both glutathione reductase and thioredoxin mRNA and proteins [35, 49], this contrasts with our current study, where we found that glutathione reducatase (GR) and thioredoxin reductase (TRXR) protein or enzymatic activity are unchanged with ErbB2 overexpression in the heart. This suggests that the antioxidant defenses induced by an ErbB ligand (neuregulin-1β) versus a receptor up-regulation (ErbB2) are similar but not identical and the reasons for this are not known. Activity assays for additional redox signaling molecules that function as antioxidants in the H9c2 *in vitro* model of ErbB2 overexpression will be performed in future studies.

We propose that ErbB2 is an important protein in certain stages of cardiac hypertrophy, potentially supporting physiological hypertrophy, while blocking pathological hypertrophy. A limitation of our study, similar to other overexpression transgenic models, is the increased level of protein expression in cardiomyocytes. However, a substantial up-regulation has occurred in mouse models that experience a threshold of oxidative stress[40] or in stages of heart failure[79]. Nevertheless, our study in the heart is confirmed by other studies in cancer cells and demonstrates that ErbB2 induces and activates two classes of protective proteins in the heart: nRTKs and antioxidant enzymes. Neonatal cardiomyocytes isolated from wild type and ErbB2^{tg} hearts will be used to more closely examine the multi-protein connection between ErbB2, Abl kinases, and GPx. For these studies, cardiomyocytes will be treated with imatinib mesylate, a c-Abl/Arg inhibitor that has been shown to reduce cardiac GPx activity[80]. We anticipate that ErbB2^{tg} cardiomyocytes treated with imatinib mesylate will have reduced GPx activity compared to untreated ErbB2^{tg} cardiomyocytes.

In summary, the heightened antioxidant capacity in the ErbB2^{tg} hearts may be one contributing factor supporting the lack of heart failure in this model of hypertrophy. It is possible that drug therapies that specifically target ErbB2 are effectively inhibiting the antioxidant defenses that are necessary to maintain normal heart function, especially under hypertrophic settings and in conditions of oxidative stress. We have shown that ErbB2 up-regulates c-Abl and Arg, which are important nRTKs for cardiac growth and development[81, 82]. In fact, drugs such as imatinib mesylate that target c-Abl/Arg are known to cause cardiac dysfunction[83-86]. Our studies are the first to show that cardiac ErbB2 up-regulates and activates GPxs in the heart. We also show that ErbB2 associates

with c-Abl and Arg and that the small molecule inhibitor of EGFR/ErbB2, lapatinib, reduces levels of c-Abl/Arg with subsequent reduction of GPx enzymatic activity. With this heightened GPx antioxidant defense, the ErbB2^{tg} hearts and their mitochondria consequently have less ROS. As a proof of principle, our *in vitro* studies provided support for the protective role of ErbB2 against doxorubicin-induced oxidative stress and cellular death. Though we did not observe any differences in GPx activity under ErbB2 overexpression and knockdown in H9c2 cells, we hypothesized that the hypetrophic phenotype is required for the increase in GPx activity that occurs in the ErbB2^{tg} hearts. Furthermore, primary neonatal cardiomyocytes will serve as the more appropriate model over H9c2 cells to assess GPx activity under the control of ErbB2 signaling. Finally, our findings suggest that adverse effects on redox signaling pathways that are necessary for maintaining antioxidant defenses may be an important mechanism of cardiac toxicity induced by drugs that target ErbB2 and Abl kinases.

Fig. 3-4. ErbB2^{tg} hearts have less oxidative stress and lower levels of $O_2^{-\cdot}$ and mitochondrial H_2O_2 compared to wild type hearts. (A) Left ventricular homogenates from ErbB2^{tg} hearts have less oxidative stress based on the oxidation of the DCF-DA fluorescent indicator of general oxidative stress (n=4 per group). (B) An EPR anaylsis using the $O_2^{-\cdot}$ -specific probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl pyrrolidine (CMH) indicates that the ErbB2^{tg} hearts contain less $O_2^{-\cdot}$ (n=3-5 per group). (C) Isolated heart mitochondria from ErbB2^{tg} hearts produce less mitochondrial H_2O_2 than the wild type hearts as determined by the Amplex Red assay (n=6 per group). (D) Representative immunoblot of protein carbonyl levels in total heart lysates do not show any differences between both groups (n=6 per group) *$p<0.05$. Values represent the means and SD.

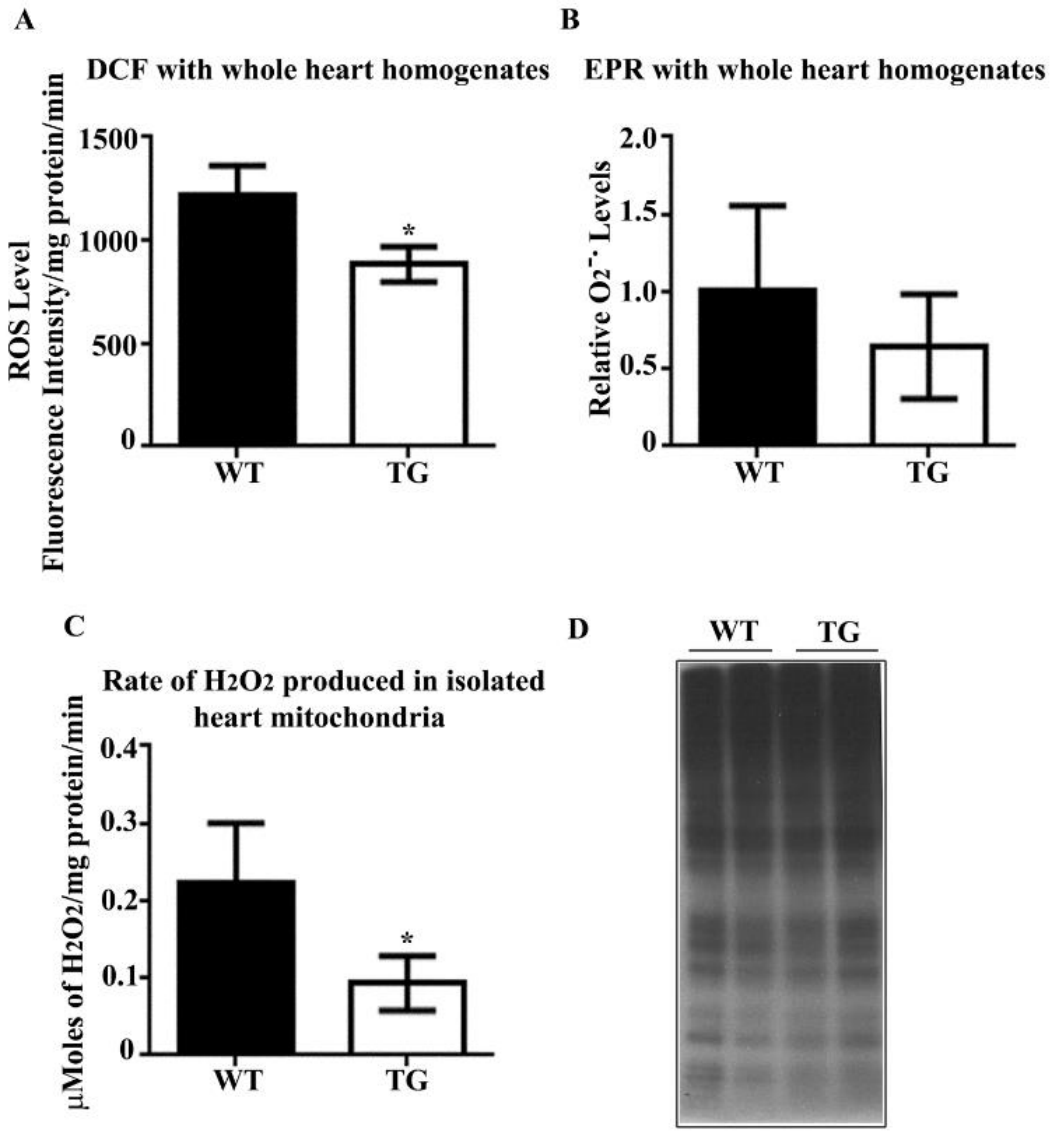
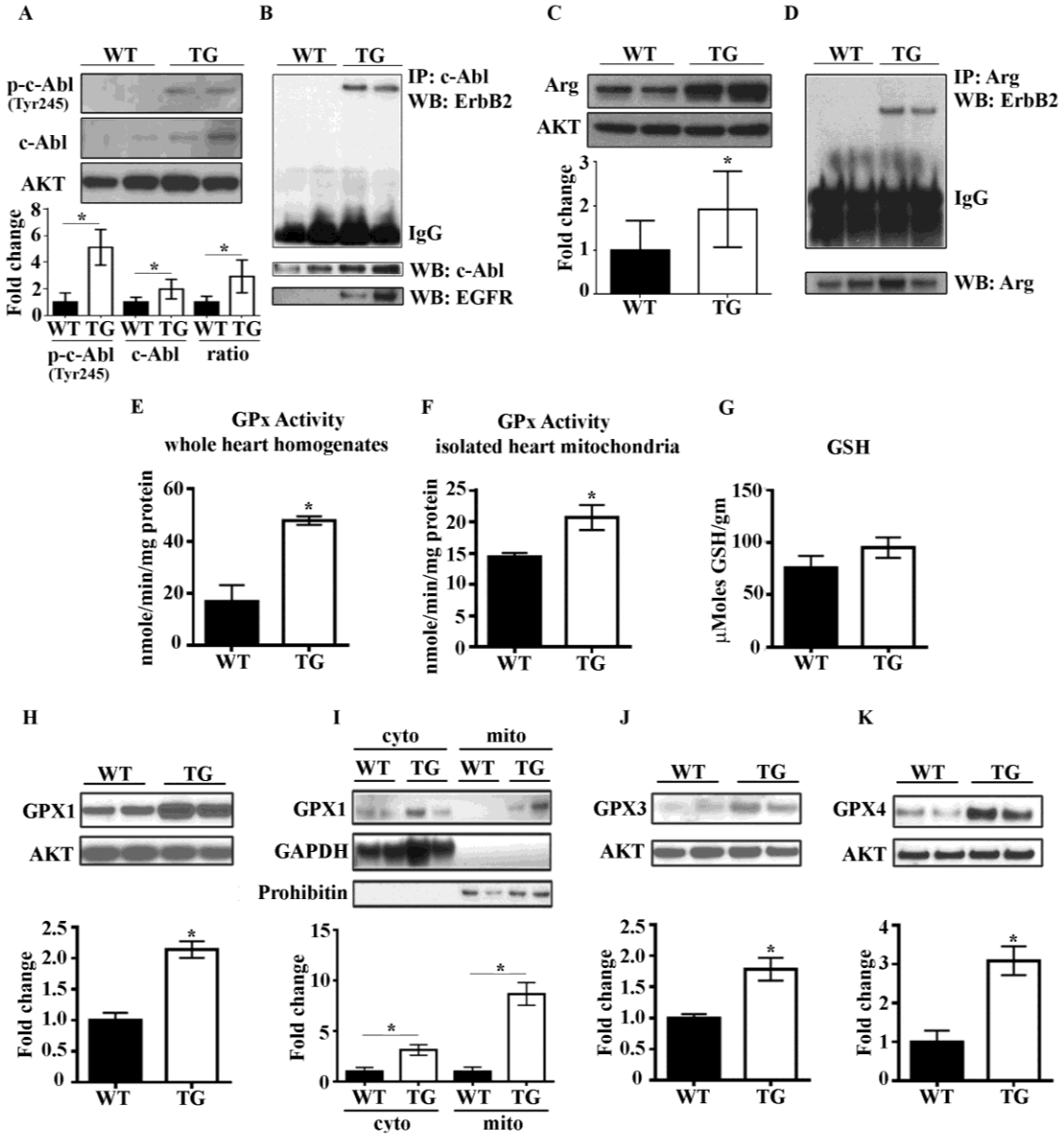

A
DCF with whole heart homogenates
ROS Level
Fluorescence Intensity/mg protein/min
1500
1000
500
0
*
WT
TG
B
EPR with whole heart homogenates
Relative O2•- Levels
2.0
1.5
1.0
0.5
0
WT
TG
C
Rate of H2O2 produced in isolated heart mitochondria
μMoles of H2O2/mg protein/min
0.4
0.3
0.2
0.1
0
*
WT
TG
D
WT
TG

Fig. 3-5. c-Abl and Arg proteins that are known to regulate glutathione peroxidase (GPx) activity are upregulated and co-immunoprecipitate with ErbB2 in the heart. Increased levels of GPx 1, 3, and 4 proteins and GPx activity are induced by cardiac ErbB2 overexpression. (A) Representative immunoblots show higher expression of phosphorylated c-Abl at Tyr245 (p-c-Abl (Tyr245)) and total c-Abl protein in ErbB2^{tg} hearts (n=7 per group). (B) c-Abl was immunoprecipitated from whole heart homogenates and co-immunoprecipitated with ErbB2 (n=6 per group) and EGFR (n=2 per group). (C) Representative immunoblot of Arg shows higher expression of Arg in the ErbB2^{tg} hearts compared to the wild type group (n=7 per group). (D) Arg was immunoprecipitated from whole heart homogenates and co-immunoprecipitated with ErbB2 (n=6 per group). (E-F) Whole heart homogenates (n=4 per group) and mitochondria (n=8 per group) isolated from the ErbB2^{tg} hearts have higher GPx activity than wild type hearts. (G) Glutathione (GSH) levels are not different between WT and TG animals (n=6 per group). (H) Representative immunoblot shows higher expression of GPx1 in whole heart homogenates of transgenic hearts compared to wild type hearts (n=12 per group). (I) GPx1 is elevated in cytosolic and mitochondrial fractions from ErbB2^{tg} hearts as shown in representative immunblots (n=6 per group). (J) Representative immunoblots show higher expression of GPx3 (n=7 per group) and (K) GPx4 in transgenic hearts compared to wild type hearts *$p<0.05$. Values represent the means and SD.


A
WT
TG
p-c-Abl
(Tyr245)
c-Abl
AKT
Fold change
p-c-Abl
(Tyr245)
c-Abl
ratio
B
IP: c-Abl
WB: ErbB2
IgG
WB: c-Abl
WB: EGFR
C
Arg
AKT
Fold change
D
IP: Arg
WB: ErbB2
IgG
WB: Arg
E
GPx Activity
whole heart homogenates
nmole/min/mg protein
F
GPx Activity
isolated heart mitochondria
nmole/min/mg protein
G
GSH
µMoles GSH/gm
H
GPX1
AKT
Fold change
I
cyto
mito
GPX1
GAPDH
Prohibitin
Fold change
J
GPX3
AKT
Fold change
K
GPX4
AKT
Fold change

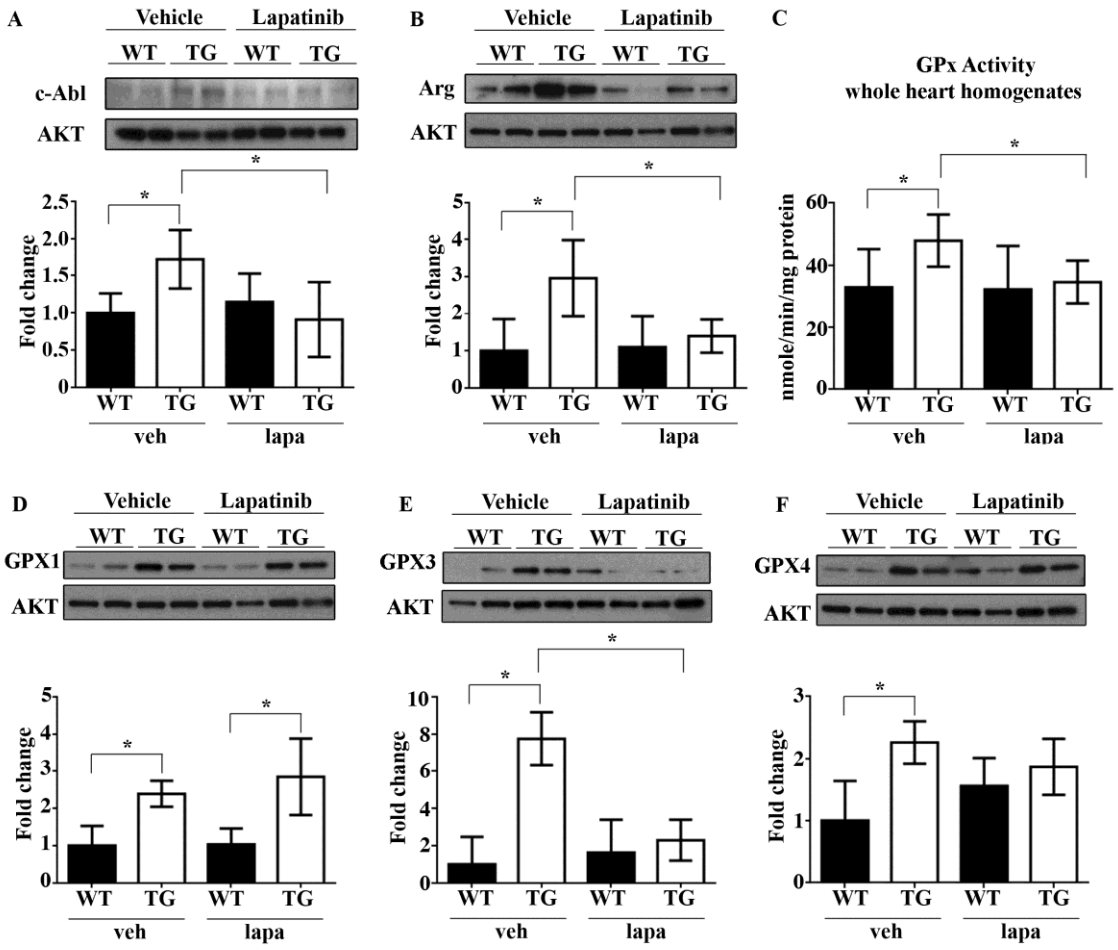


Fig. 3-6. ErbB2 and EGFR kinases are responsible for maintaining c-Abl and Arg protein levels and GPx activity. (A) Treatment with the ErbB2/EGFR inhibitor lapatinib reduces c-Abl protein in transgenic whole heart homogenates (n=6 per group). (B) Lapatinib reduces Arg protein in whole heart homogenates from transgenic animals (n=4-5 per group). (C) GPx activity is reduced by lapatinib in the transgenic animals (n=7 per group). (D-F) Immunoblots show that only GPx3 protein is reduced in transgenic animals after treatment with lapatinib (n=4 per group). * $p<0.05$, veh=vehicle, lapa=lapatinib. Values represent the means and SD.

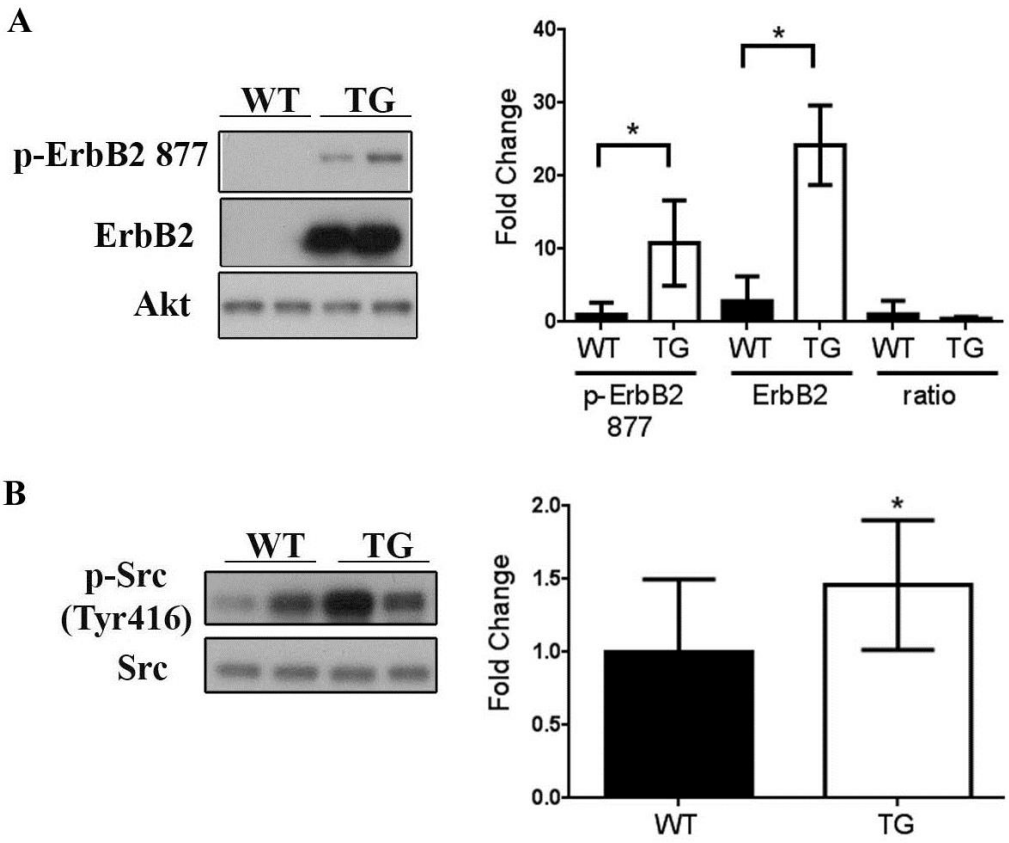


Fig. 3-7. Expression of p-ErbB2, Src, and p-Src proteins in the ErbB2^{tg} mouse hearts. ErbB2^{tg} mouse hearts have higher levels of (A) phosphorylated ErbB2 (p-ErbB2) and (B) Src (p-Src) proteins compared to wild type hearts (n=6 and 10 per group, respectively). *$p<0.05$. Values represent the means and SD.

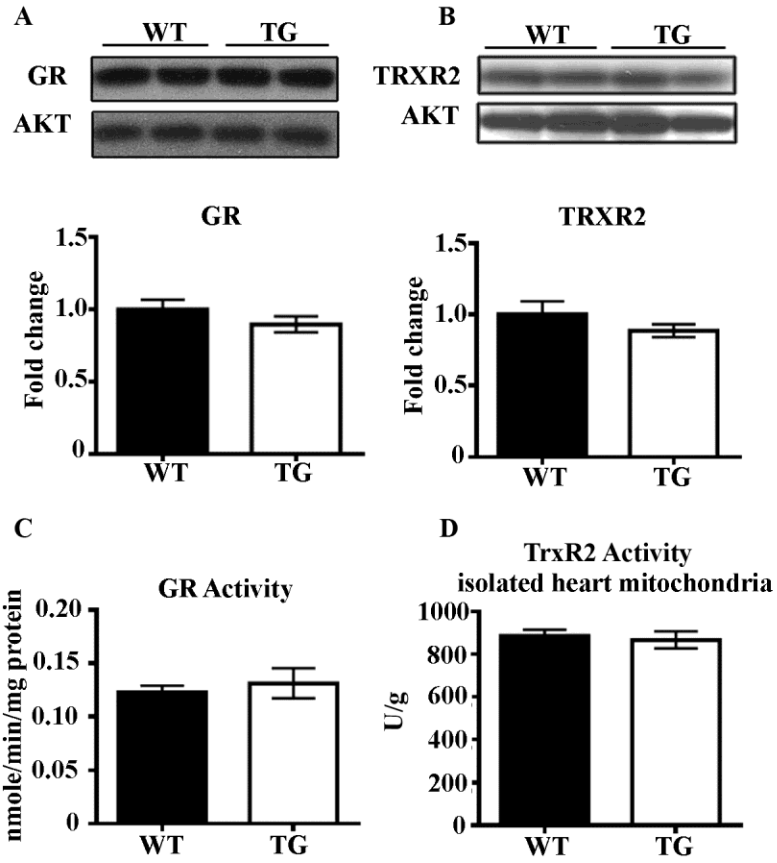
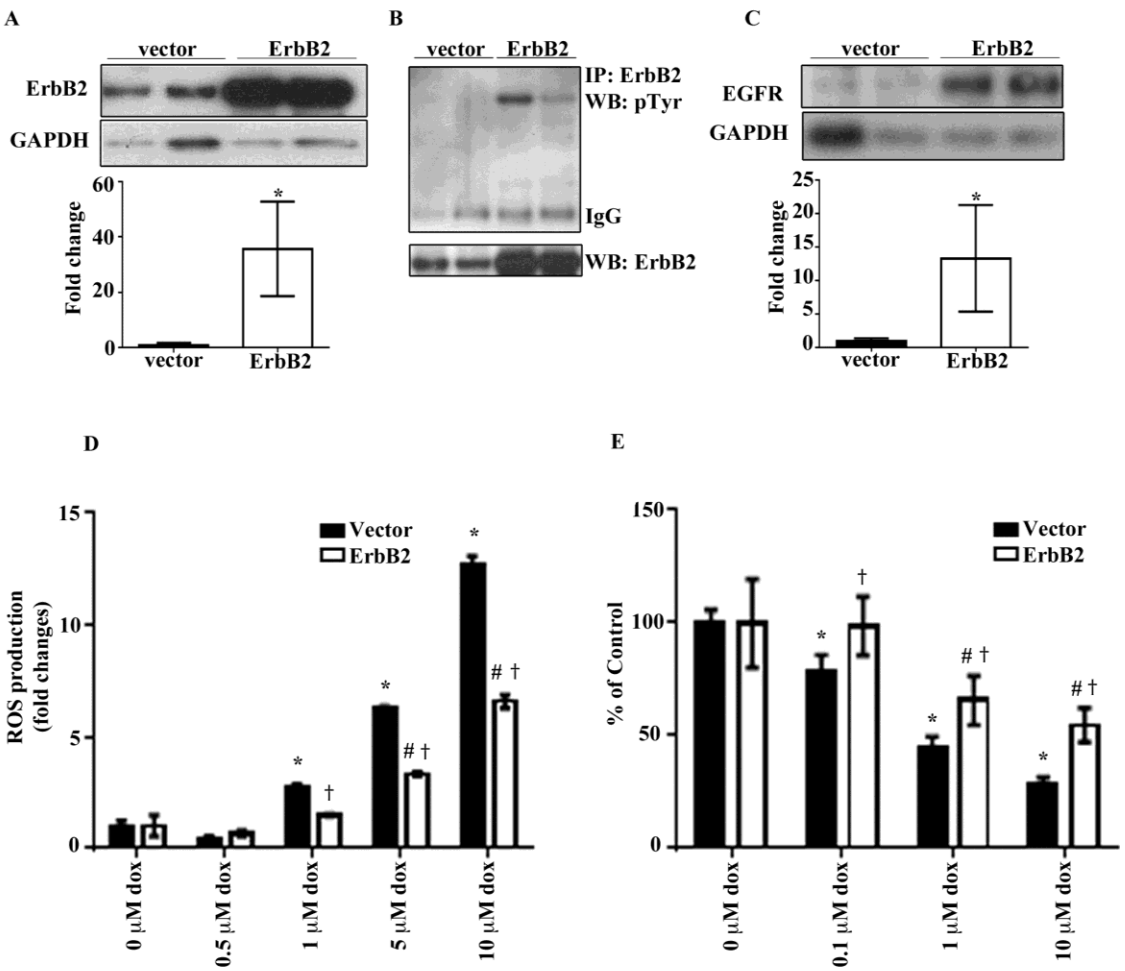


Fig. 3-8. Glutathione reductase (GR) and thioredoxin reductase 2 (TRXR2) protein levels and activity are unchanged by overexpression of ErbB2 in the heart. (A) GR protein levels (n=7 per group) and (C) activity (n=7 per group) are not different between the WT and TG hearts. (B) No difference in TRXR2 protein levels (n=5 per group) or (D) activity (n=6 per group) was found between both groups of animals. *$p<0.05$. Values represent the means and SD.

Fig. 3-9. ErbB2 overexpression in H9c2 cells protects against doxorubicin-induced oxidative stress and cell death. H9c2 cells were transfected with pcDNA (3.1) + vector or ErbB2 plasmid. (A) A representative immunoblot shows that transfection with ErbB2 plasmid increases the levels of total and (B) phosphorylated ErbB2 proteins (n=6 per group). (C) ErbB2 transfection in H9c2 cells upregulates EGFR protein as shown by a representative immnublot (n=6 per group). (D) After 48 hr of transfection the cells were incubated with vehicle or doxorubicin (dox) for 6-24 hr. ROS production was determined using the DCF-DA fluorescent assay for general oxidative stress (n=6 per treatment group) and (E) cell viability was determined by the MTT assay (n=6 per treatment group). *$p<0.05$. Two-way ANOVA<0.05, *$p<0.05$ versus vector with 0μM dox, #$p<0.05$ versus ErbB2 with 0μM dox, ┼$p<0.05$ versus vector with dox dose. Values represent the means and SD.


A
vector
ErbB2
ErbB2
GAPDH
Fold change
60
40
20
0
*
vector
ErbB2
B
vector
ErbB2
IP: ErbB2
WB: pTyr
IgG
WB: ErbB2
C
vector
ErbB2
EGFR
GAPDH
Fold change
25
20
15
10
5
0
*
vector
ErbB2
D
Vector
ErbB2
ROS production
(fold changes)
15
10
5
0
*
†
†
0 μM dox
0.5 μM dox
1 μM dox
5 μM dox
10 μM dox
E
Vector
ErbB2
% of Control
150
100
50
0
*
†
†
0 μM dox
0.1 μM dox
1 μM dox
10 μM dox

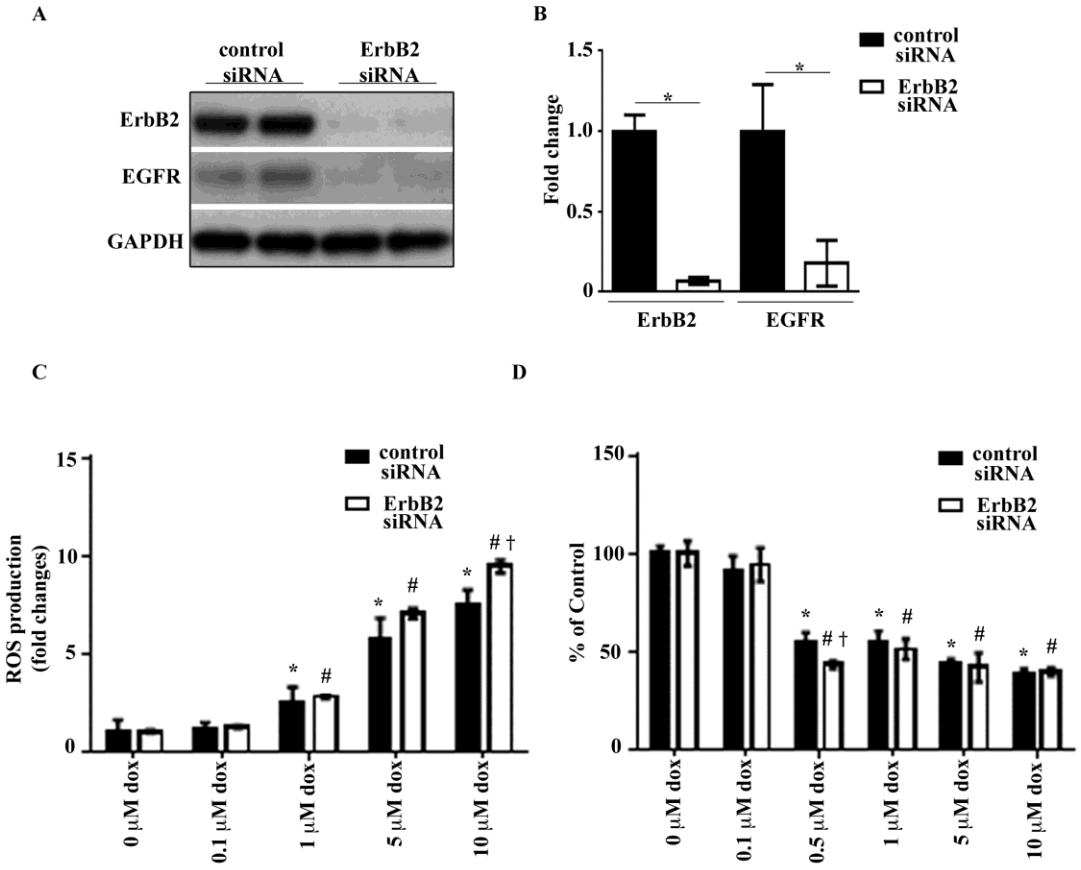


Fig. 3-10. siRNA-mediated knockdown of ErbB2 in H9c2 cells reduces the protective effect against doxorubicin-induced oxidative stress and cell death. (A) Transfection with ErbB2 siRNA decreases the levels ErbB2 and (B) EGFR proteins as shown by representative immunoblots (n=6 per group). (C) After 48 hr of transfection the cells were incubated with vehicle or doxorubicin (dox) for 6-24 hr. ROS production was determined using the DCF-DA fluorescent assay for general oxidative stress (n=6 per treatment group) and (E) cell viability was determined by the MTT assay (n=6 per treatment group). *p<0.05. Two-way ANOVA<0.05, *p<0.05 versus vector with 0μM dox, #p<0.05 versus ErbB2 with 0μM dox, ┼p<0.05 versus vector with dox dose. Values represent the means and SD.

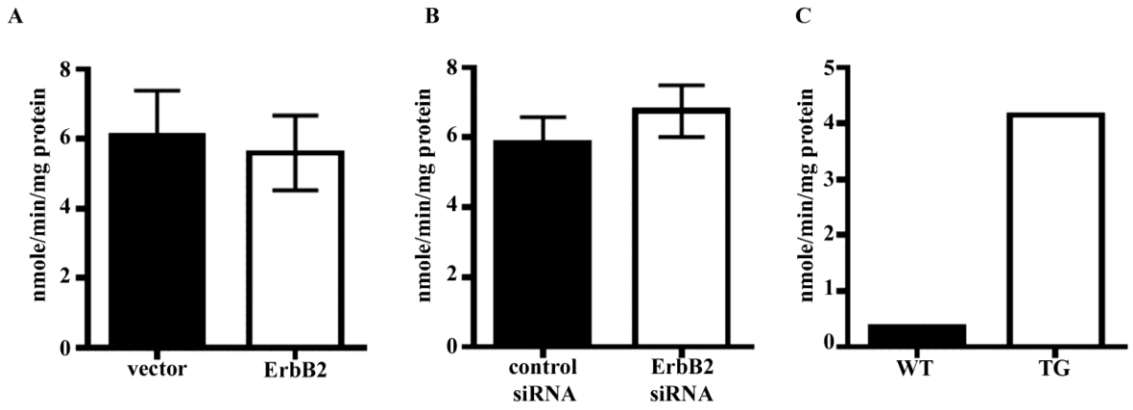


Fig. 3-11. GPx activity is not altered with overexpression or knockdown of ErbB2 in H9c2 cells. Preliminary data from isolated neonatal cardiomyocytes indicate that the ErbB2^{tg} hearts have higher GPx activity compared to wild type cardiomyocytes. (A) H9c2 cells transfected with plasmid to overexpress ErbB2 or (B) siRNA to knockdown ErbB2 have no effect on GPx activity (n=6 per group). (C) Neonatal cardiomyocytes isolated from the ErbB2^{tg} hearts have greater GPx activity than WT cardiomyocytes (n=1 per group, pooled cardiomyocytes from each group). Values represent the means and SD.

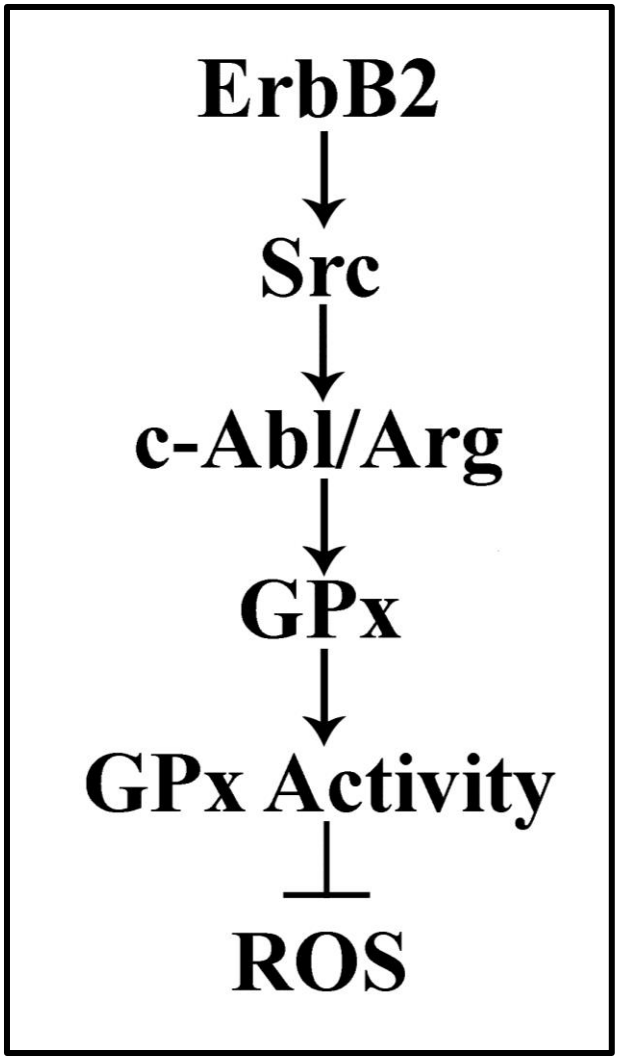


Fig. 3-12. Proposed mechanism of ErbB2 signaling through Src and the nonreceptor tyrosine kinases c-Abl and Arg that will subsequently activate GPx for the removal of ROS.

3.5 Methods and Materials

Animals

This study was performed in strict accordance with the recommendations in the "Guide for the Care and Use of Laboratory Animals" (2011) of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions (Animal Welfare Assurance # A-3273-01). The ErbB2^{tg} and wild type [48] mice used in this study were developed as described [33].

Cell Culture

The H9c2 rat cardiomyoblast cell line was obtained from ATTC (USA) and maintained in DMEM supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotic at 37ºC and 5% CO_2. The cells were subcultured at a ratio of 1:4 every 3-4 days.

ErbB2 overexpression and siRNA knockdown of ErbB2 in H9c2 cell line

H9c2 cells were transfected with rat-neu/ErbB2 or pcDNA 3.1(+) control plasmid DNA using the Fugene HD (Promega, Madison, WI) reagent. After 48 hr the cells were harvested to obtain lysates for subsequent assays or treated with vehicle/doxorubicin (Bedford Laboratories, Bedford, OH) for MTT and DCF assays. For the siRNA experiments, ON-TARGET plus Rat ErbB2 (24337; Thermo Scientific, Waltham, MA) siRNA with the targeting sequences GGUAGACGCUGAAGAGUAU and GACAGAAGAUCCGGAAGUA were used at 25nM.

Treatment of H9c2 cells with doxorubicin

Dosages for doxorubicin were determined according to published studies of doxorubicin-treated H9c2 cells[75]. For ErbB2 overexpression and knockdown studies, doxorubicin was

added to the H9c2 cells at 24 and 48hr-post transfection. Assays for oxidative stress and cellular viability were performed 6-24hr after drug treatment.

Isolation of neonatal cardiomyocytes

Wild type and ErbB2^{tg} neonate mice between the ages of one- to two-days-old were used for cardiomyocyte isolations[87]. Briefly, the mice were euthanized via decapitation and the hearts were immediately removed and rinsed in HBSS without Ca^{2+} and Mg^{2+} (Life Technologies, Grand Island, NY), minced, and enzymatically dissociated in 0.25% trypsin (Life Technologies, Grand Island, NY) containing HBSS without Ca^{2+} and Mg^{2+}. The supernatant containing the cells was collected and HBSS with Ca^{2+} and Mg^{2+} was added. After the tissue was dissociated, the cells were collected and resuspended in complete MEM (Life Technologies, Grand Island, NY) media containing 20% FBS, 100U/ml penicillin, and 100µg/ml streptomycin. The cells were pre-plated for 2hrs to exclude non-muscle cells and plated at the appropriate cell density.

Microarray Analysis of Wild Type and Transgenic Mice

Total RNA was extracted from the hearts of the WT and ErbB2 TG mice (3 mice per genotype) [33]for whole genome expression analysis using an Illumina Bead Array. Microarray data were analyzed using a spreadsheet-based microarray analysis program (DIANE 6.0) and the oxidative stress genes were analyzed by cluster analysis to determine the patterns of expression between ErbB2 TG mice and WT mice. Genes were determined to be differentially expressed based on the z-ratio (Z ratio > 1.5), false discovery rate (fdr< 0.3) and statistical significance at $p < 0.05$.

Real-time PCR and Primers Design

Total RNA was isolated from the hearts of the WT and ErbB2 TG mice (8 mice per genotype) as described [33]. Glutathione peroxidase 1 (Gpx1) and glutathione peroxidase 3 (Gpx3) were chosen from the microarray data of statistically significantly up-regulated oxidative stress genes and were evaluated by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR). The primer pairs for mouse Gpx1 were as follows: 5'-CGGGGCAAGGTGCTGCTCAT-3' forward and 5'-GCACGGGAAACCGAGCACCA-3' reverse. The primer pairs for mouse Gpx3 were as follows: 5'-CATCCTGCCTTCTGTCCCTG-3' forward and 5'-GTTGGAAGGGAAGCCCAGAA-3' reverse. Peptidylprolylisomerase A (PPIA) was used for RNA normalization. The qRT-PCR results were calculated by delta-delta Ct method using the mean of the delta Ct value of wild type mice as a normalization factor.

Immunoblotting

Transfected H9c2 cells were washed with cold 1X PBS and harvested in 1X PBS with a cell scraper and centrifuged. Lysis buffer (25 mM Na_2HPO_4, 25 mM NaH_2PO_4, 150 mM NaCl, 10% glycerol, 1 mM EDTA) was added to the cell pellet and spun down. The final supernatant contained the soluble protein used for standard gel electrophoresis and immunoblotting[33].

Both WT and TG hearts were evaluated by Western blotting for protein expression. Frozen left ventricle (40 mg) was rapidly homogenized in 200–300 µL of lysis buffer (25 mM Na_2HPO_4, 25 mM NaH_2PO_4, 150 mM NaCl, 10% glycerol, 1 mM EDTA) and standard gel electrophoresis and immunoblotting were performed[33]. The primary antibodies used were directed against GPx1, GPx3, GR (Abcam, Cambridge,

Massachusetts), GPx4 (Cayman Chemical, Ann Arbor, MI), Akt, c-Abl, p-c-Abl (Tyr245), p-ErbB2 (Tyr877), p-Src (Tyr416), Src (Cell Signaling, Danvers, MA), ErbB2, EGFR, TrxR2, Prohibitin, GAPDH (Santa Cruz, Santa Cruz, CA), and Arg (US Biological, Salem, MA). After incubation in anti-rabbit, -mouse, or -goat (GE Healthcare, Piscataway, NJ and Santa Cruz Biotechnology, Santa Cruz, CA) horseradish peroxidase-linked secondary antibody, blots were exposed to chemiluminescent substrate (Pierce, Rockford, IL) and exposed to CL-Xposure film (Pierce, Rockford, IL). Levels of Akt[33] and GAPDH proteins were measured to normalize protein quantities across samples.

Immunoprecipitation

Lysates from H9c2 cells and heart tissue were prepared for immunoprecipitation as described [33]. Briefly, cell lysates were incubated with anti-ErbB2 antibody (Santa Cruz, Santa Cruz, CA), anti-Src antibody (Cell Signaling, Danvers, MA), anti-c-Abl antibody (Santa Cruz, Santa Cruz, CA), or Arg antibody (US Biological, Salem, MA) at 4°C overnight (3 μg of antibody per 1000 μg of total protein). Protein A or G beads (GE Healthcare Life Sciences, Pittsburgh, PA) were washed with cold lysis buffer and centrifuged (3,000 RPM for 1 min). Tissue lysates were incubated with anti-c-Abl antibody (Santa Cruz, Santa Cruz, CA) or Arg antibody (US Biological, Salem, MA) at 4°C overnight (3 μg of antibody per 1000 μg of total protein). Protein A or G beads (GE Healthcare Life Sciences, Pittsburgh, PA) were washed with cold lysis buffer and centrifuged (3,000 RPM for 1 min). The buffer was aspirated and the beads were added to the antibody-treated lysates and incubated at 4°C overnight. The beads were washed three times and centrifuged at 3,000 RPM for 1 minute. The supernatant was discarded and urea buffer with beta-mercaptoethanol (BME) was added to the beads. The

suspension was heated at 95°C for 5 minutes and centrifuged at 3,000 RPM for 1 minute. The supernatant was loaded on a SDS gel and the immunoblotting protocol was followed as described above. The membranes were probed with phospho-tyrosine **(**Cell Signaling, Danvers, MA) or anti-ErbB2 antibody (Santa Cruz, Santa Cruz, CA).

Mitochondrial Isolation from Hearts

Mitochondria were isolated from hearts as described in[88]. Briefly, hearts were excised from TG and WT mice and placed in mitochondrial isolation buffer (10 mM Hepes, 0.25 M Sucrose, 1 mM EGTA) to remove excess blood from the hearts as described[88]. The hearts were cut into small pieces and then placed in a 30 ml homogenizer with 0.7 mg/mL (per mouse heart) Nagarse, Protease Type XXIV from *Bacillus licheniformis* (Sigma-Aldrich, St. Louis, MO) in 1 ml of mitochondrial isolation buffer. The tissue was homogenized for approximately eight minutes, transferred to a 1.5 mL microcentrifuge tube and centrifuged at 8,500 *x g* for eight minutes. The supernatant containing the protease was removed immediately and the remaining pellet was homogenized in fresh mitochondrial isolation buffer without the protease. The contents were transferred to 1.5 mL micocentrifuge tubes and a series of differential centrifugations at 4°C were performed to isolate the heart mitochondria and remove excess cellular components and tissue debris [88]. The final mitochondrial pellet was re-suspended in the mitochondrial resuspension buffer (10 mM Hepes, 0.25 M Sucrose, 0.1% BSA) and protease inhibitors (Roche Applied Science, Indianapolis, IN) and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich, St. Louis, MO) were added at a 100-fold dilution.

Reactive Oxygen Species Measurements

The formation of ROS in heart homogenates and transfected H9c2 cells (1.2×10^4 cells per well in a 96-well plate) was measured using the 2,7-dichlorodihydrofluorescein diacetate (DCF-DA, Sigma-Aldrich, St. Louis, MO) general oxidative stress fluorescent indicator. For heart homogenates, 1μM of DCF-DA was incubated with 200μg of heart homogenate and fluorescence was recorded over 1hr at an excitation of 485 nm and 522 nm emission[89]. Measurements of ROS in H9c2 cells were performed with 50μM DCF-DA that was prepared in clear DMEM and added to each well for 30 min at 37ºC in the dark. An initial reading was taken at an excitation of 485 nm and 522 nm emission. The DCF-DA was removed and vehicle or doxorubicin was added to each well. Readings were taken at an excitation of 485 nm and 522 nm emission every hour for up to 6 hr followed by a 24 hr reading. The Amplex Red assay (Invitrogen, Carlsbad, CA) was used to measure hydrogen peroxide (H_2O_2) produced from the isolated heart mitochondria. The mitochondria were energized with succinate [90, 91] and H_2O_2 production was measured in both unstimulated (-ADP) and stimulated (+ADP) states. For preparation of tissue for electron paramagnetic resonance spectroscopy (EPR), ventricles were homogenized in lysis buffer (12.5 mM Na_2HPO_4, 12.5 mM NaH_2PO_4, 75 mM NaCl, 10% glycerol, 0.5 mM EDTA, 1:100 of phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich, St. Louis, MO), one tablet of complete mini, EDTA-free protease inhibitor (Roche Applied Science, Indianapolis, IN)) and centrifuged at 21,000 *x g* for 10 minutes. Stock solutions of 1-hydroxy-3- methoxycarbonyl-2,2,5,5- tetramethylpyrrolidine (10mM CMH, Enzo Life Sciences, Farmingdale, NY) were made in nitrogen purged 0.9% (w/v) NaCl, 25g/L Chelex 100 (Bio-Rad) and 0.1mM DTPA [92]. 70 μg of protein were combined with the

CMH probe (1mM). Samples were assayed in 50 μL glass capillary tubes at room temperature using the Bruker E-scan EPR spectrometer (Bruker, Billerica, MA). Spectrometer settings were as follows: sweep width: 100G; microwave frequency: 9.75 GHz; modulation amplitude: 1 G; conversion time: 5.12ms; time constant: 5.12ms; receiver gain, 2×10^2; number of scans, 4. Relative superoxide levels were assessed by measuring the signal amplitude.

Measurement of Oxidized Proteins

Protein carbonyl levels were measured in lysates from left ventricular tissue using the OxyBlot Protein Oxidation Detection Kit (Millipore, Billerica, MA) according to the manufacturer's protocol. Briefly, 20 μg of protein were derivatized to 2, 4-dinitrophenylhydrazone (DNP-hydrazone) with 2, 4-dinitrophenylhydrazine (DNPH). The protein samples were separated on a SDS gel using the immunoblotting protocol as described above. The membrane was probed with rabbit anti-DNP antibody (provided in the kit) and the blots were subsequently processed based on the immunoblotting instructions described above.

MTT Assay

Cell viability was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay whereby cells with an active mitochondrial dehydrogenase convert MTT to an insoluble purple formazan. Briefly, transfected H9c2 cells (1.2×10^4 cells per well in a 96-well plate) were exposed to vehicle or doxorubicin with or without 5mM N-acetylcysteine (NAC, Sigma-Aldrich, St. Louis, MO) for 6 hr. The media was aspirated and 100 μl of 5 mg/ml MTT reagent in PBS was added to each

well at 37ºC for 4 hr. The insoluble purple formazan was dissolved in 100 µl of dimethyl sulfoxide and the absorbance was read at 570 nm on a microplate reader.

Lapatinib Treatment

Wild type or ErbB2^{tg} male mice were treated with oral lapatinib (160 mg/kg) daily [93, 94]. 24G-20 gavage needles (Braintree Scientific, Inc., Braintree, MA) were used. Lapatinib (LC Laboratories, Woburn, MA) suspension was made fresh before each treatment. Lapatinib was diluted with buffer containing 0.5% carboxymethyl cellulose, 1.8% sodium chloride, and 0.4%Tween-80 in deionized water (dH_2O). Because a percentage of unmetabolized lapatinib is excreted with feces, mice were housed in separate cages (vehicle or lapatinib group). Animals were treated daily for 11 days and then euthanized 2 hours after the final treatment. Hearts were excised, weighed, and tibia length measured. Hearts were sectioned and the left ventricle frozen for further molecular studies.

Glutathione Peroxidase (GPx) Activity Assay

GPx activity was measured with a NADPH-linked enzymatic assay to monitor the decrease in NADPH absorbance at 340 nm. Reagents were obtained from the GPx activity assay kits ADI-900-158 (Enzo Life Sciences, Plymouth Meeting, PA) and GPx Assay Kit 703102 (Cayman Chemical, Ann Arbor, MI). Lysates from transfected H9c2 cells were washed with cold 1X PBS and harvested in 1X PBS with a cell scraper and centrifuged. Lysis buffer (25 mM Na_2HPO_4, 25 mM NaH_2PO_4, 150 mM NaCl, 10% glycerol, 1 mM EDTA) was added to the cell pellet and spun down. Hearts from WT and TG mice were homogenized in cold assay buffer containing 0.4 mM PMSF, protease inhibitors, and 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and the homogenate

was centrifuged at 10,000 *x g* for 10 minutes at 4°C. The supernatant was collected and diluted in assay buffer. Mitochondria were isolated as described above and were similarly diluted in the GPx assay buffer.

Quantification of Glutathione

Glutathione was quantified as described [95] with slight modifications using a Bioxytech GSH/GSSG-412 kit (Oxis Research, Portland, OR). Hearts were homogenized in six volumes of 5% metaphosphoric acid and 5.6 µl of the supernatant were added to 394.4 µl of assay buffer (100 mmol/l $NaPO_4$, 5 mmol/l EDTA, pH 7.5). After the addition of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and glutathione reductase the samples were incubated for five minutes at room temperature. NADPH was added to the samples and the absorbance was recorded at 412 nm for three minutes.

Glutathione Reductase (GR) Activity Assay

GR activity was measured by the rate of NADPH oxidation at an absorbance of 340 nm. Reagents were obtained from a GR activity assay kit 703202 (Cayman Chemical Company, Ann Arbor, MI). Hearts from TG and WT mice were homogenized in cold buffer containing 50 mM potassium phosphate, 1 mM EDTA and the homogenate was centrifuged at 10,000 *x g* for 15 minutes at 4°C. The supernatant was collected and diluted in assay buffer containing 50 mM potassium phosphate, 1 mM EDTA, and 1 mg/ml BSA.

Thioredoxin Reductase 2 (TrxR2) Activity Assay

Mitochondria were isolated from the hearts of WT and TG mice. Prior to the assay, the mitochondrial membranes were removed by a series of freeze-thaw cycles between dry ice and ethanol at 37°C. After the membranes were removed, the mitochondria

preparation was centrifuged for two minutes at 20,000 *x g* and the supernatant was collected for use in the assay. The samples were prepared in a master mix containing 5mM DTNB, 8% ethanol, 300 μM NADPH, and PE buffer (100 mM potassium phosphate, 2 mM EDTA, pH 7.0). TrxR2 activity was measured by the reduction of DTNB by TrxR2 and NADPH at an absorbance of 412 nm.

Statistical Methods

GraphPad Prism software (GraphPad, La Jolla, CA) was used to perform statistical analysis. After determining means and standard deviations, the Student unpaired T-test was used to determine significance of differences between groups. Two-way analysis of variance (ANOVA) and Tukey's post hoc tests were performed for multiple comparisons. P-values of <0.05 were accepted as a significant difference.

Chapter 4

Characterization of cardiac mitochondria isolated from the ErbB2^{tg} mice

4.1 Abstract

The rationale for studying the ErbB2^{tg} heart mitochondria was based on microarray analyses that revealed a subset of genes involved in complex I of the electron transport chain were downregulated in the hearts of the ErbB2^{tg} mice. Since mitochondrial alterations have been implicated in cardiac hypertrophy it was plausible that overexpression of ErbB2 in the heart would modulate mitochondrial function in response to hypertrophy. We probed for key factors involved in mitochondrial biogenesis and found that the levels of protein in the ErbB2^{tg} hearts were unaffected by ErbB2 overexpression. Next, we performed functional studies with isolated heart mitochondria and determined that mitochondrial oxygen consumption with complex I and II substrates was not different in the ErbB2^{tg} heart mitochondria compared to wild type mitochondria. Complex I enzymatic activity of the ErbB2^{tg} heart mitochondria was similarly unaffected by ErbB2 overexpression. The discordancy between the microarray data and our mitochondrial studies requires further investigation. A known consequence of cardiac hypertrophy is altered ATP production. We measured ATP production with mitochondrial substrates for complex I, II, and IV and found that the mitochondria from the ErbB2^{tg} hearts did not have reduced ATP production compared to wild type heart mitochondria. Further characterizations of the mitochondria involved measuring the mitochondrial membrane potential ($\Delta\varphi$) with the tetramethylrhodamine ethyl ester (TMRE) fluorescent indicator. Our studies indicated that mitochondria from the ErbB2^{tg} hearts have a higher baseline $\Delta\varphi$ that was supported with additional assays from neonatal cardiomyocytes that had higher staining with a JC-1 dye that accumulates in polarized mitochondria. Since the $\Delta\varphi$ is a marker of cellular viability, our findings support the pro-

survival phenotype that is characteristic of the ErbB2^{tg} mouse model. However, additional mechanisms that may influence the $\Delta\varphi$ in the ErbB2^{tg} mice have yet to be elucidated. Lastly, we measured the calcium (Ca^{2+}) retention capacity of the mitochondria and found that mitochondria from the ErbB2^{tg} hearts have less Ca^{2+} uptake compared to wild type heart mitochondria. Though the implications for these findings are currently unknown and require further investigation, we have found that ErbB2^{tg} hearts have higher baseline Ca^{2+} transients and faster Ca^{2+} reuptake. Studies to characterize the ErbB2^{tg} heart mitochondria are far from complete; however, our findings, particularly from the $\Delta\varphi$ and Ca^{2+} assays provide a basis for exploring the mitochondria further in order to gain a better understanding for how ErbB2 regulates mitochondrial function in cases of hypertrophy without progression to heart failure.

4.2 Introduction

Initial microarray studies that compared differentially expressed genes between the wild type and ErbB2 transgenic mice revealed possible mitochondrial dysfunction caused by cardiac ErbB2 overexpression. Though the microarray analysis required further validation, it was possible that the ErbB2-induced hypertrophic phenotype altered the mitochondria, perhaps as a compensatory response to the hypertrophy. The important role of ErbB2 on mitochondrial function has been investigated in cancer biology and in various cardiac studies. For example, ErbB2 was found to translocate into the mitochondria in order to regulate mitochondrial respiratory functions that are altered in cancer cells[10]. Notably, this particular study provided insight on how mitochondrial ErbB2 contributes to chemotherapy resistance (i.e. trastuzumab/Herceptin) and the switch from oxidative phosphorylation to aerobic glycolysis—referred to as the Warburg

effect—that is advantageous for the survival of the cancer cell[10]. While the exact mechanism for the effects of mitochondrial ErbB2 on cellular metabolism is poorly understood, other studies have shown that ErbB2 regulates mitochondrial function through uncoupling protein 2 (UCP2) in cancer cells [96]. As discussed in section 1.1.3 of the first chapter, inhibition of ErbB2 in NRVM results in cellular death through apoptosis and ROS that are mediated by a decrease in $\Delta\varphi$ and impaired mitochondrial function. Collectively, these studies suggest that ErbB2 tyrosine kinase activity is important for maintaining the protective cellular adaptations through the mitochondria.

While it is known that cardiac hypertrophy alters mitochondrial function, the degree of mitochondrial impairment is dependent upon whether the hypertrophy is physiological or pathological. In physiological hypertrophy, such as when the heart enlarges in response to pregnancy and exercise, the heart has the ability to adapt to the increased workload without negatively affecting cardiac function[97-100]. Conversely, in pathological hypertrophy, the negative hypertrophic response can result in contractile dysfunction and heart failure[98, 101]. Since we observed hypertrophy without progression to heart failure in the ErbB2 transgenic model, we hypothesized that mitochondria from the transgenic hearts would have features that are characteristic of physiological hypertrophy. Listed in Table 4-1 are the mitochondrial alterations that have been identified in both physiological and pathological hypertrophies and their corresponding stimuli to induce hypertrophy [98]. Though various signaling transduction pathways have been implicated in altering mitochondrial function during physiological and pathological hypertrophies, the mechanisms involved in these pathways are not completely understood [98]. Interestingly, the ErbB2 transgenic mice display a few of the features that are described for

pathological hypertrophy, such as fibrosis and upregulated fetal genes that are found in the microarray analysis. However, much of the data from the ErbB2 transgenic model suggest that the transgenic mice lack many of the characteristics of mitochondrial dysfunction that are listed in Table 4-2 [98] that are correlated with pathological hypertrophy, such as increased ROS. Taken together, our findings support the idea that some overlap exists between the pathophysiology of physiological and pathological hypertrophy.

Due to the complexity of the mitochondria, we chose to examine several mitochondrial indices that would serve as an initial basis for subsequent, in-depth analyses on the effects of ErbB2 overexpression on the mitochondria. We examined differences in the expression of mitochondrial biogenesis proteins and measured mitochondrial respiratory function with complex I and II substrates of the electron transport chain. Additional studies to characterize the mitochondria included measurements of ATP,$\Delta\varphi$, and calcium retention capacity. Our findings will allow us to identify potential novel mechanisms of ErbB2 signaling to the heart mitochondria, whereby alterations in mitochondrial function are uniquely regulated by cardiac ErbB2 overexpression and the ensuing hypertrophy.

	Physiological hypertrophy	Pathological hypertrophy
Stimuli	Volume overload (isotonic exercise) Pressure overload (isometric exercise) Pregnancy	Volume overload (valvular disease, aortovenous fistula) Pressure overload (arterial hypertension, aortic constriction)
Foetal gene expression	Normal	Up-regulated
Cardiac function	Normal/improved	Increasingly impaired during the time course
Decompensation	Not occurring	Occurring
Cardiac structure	Increased myocyte volume Formation of new sarcomeres	Increased myocyte volume Formation of new sarcomeres Interstitial fibrosis Increased rates of apoptosis
Fatty acid oxidation	Unchanged or increased	Decreased
Glucose oxidation	Unchanged or increased	Unchanged Maybe reduced during heart failure
Mitochondrial biogenesis	In accordance to cellular hypertrophy	Unchanged during compensated hypertrophy Maybe diminished during heart failure
ATP production	Sufficient	Impaired

Table 4-1. Characteristics of physiological and pathological hypertrophy. The hypertrophic stimuli and the corresponding mitochondrial alteration is shown for each type of hypertrophy.

Potential mechanisms of mitochondrial dysfunction in pathological hypertrophy
ROS
Altered ANT expression
Cardiolipin loss or peroxidation
Fatty acid and lipid overload
Mitochondrial uncoupling
Impaired mitochondrial biogenesis
Reduced transcriptional signaling of regulators of mitochondria

Table 4-2. Mechanisms of mitochondrial dysfunction in pathological hypertrophy. Pathological cardiac hypertrophy may involve multiple mechanisms that contribute to mitochondrial dysfunction.

4.3 Results

Mitochondrial biogenesis proteins are unchanged by cardiac ErbB2 overexpression

The initial efforts to characterize the mitochondria involved an assessment of whether key mitochondrial biogenesis proteins were differentially expressed between the wild type and ErbB2 transgenic hearts. One of the most important activators of mitochondrial biogenesis is the peroxisome proliferator-activated receptor-γ (PPARγ) coactivator-1 (PGC-1) family that consists of two members: PGC-1α and PGC-1β [102, 103]. These proteins function as co-transcriptional regulation factors that stimulate mitochondrial biogenesis by activating transcription factors such as the nuclear respiratory factors (NRF-1 and NRF-2) that will subsequently induce the expression of mitochondrial transcription factor A (Tfam), which is essential for mitochondrial DNA (mtDNA) transcription and replication [102, 103]. In addition to the NRFs, PGC-1α regulates mitochondrial biogenesis via the uncoupling proteins (UCPs) [102, 103]. Another key regulator of mitochondrial biogenesis that was assessed in our studies is adenosine monophosphate-activated protein kinase (AMPK) that functions primarily to regulate intracellular energy metabolism, particularly during changes in cellular energetics [102]. A third mitochondrial biogenesis protein that we measured was the orphan nuclear receptor estrogen-related receptor α (ERRα) that is activated by PGC-1α in order to regulate the expression of genes involved in oxidative phosphorylation and mitochondrial biogenesis [102-104]. Lastly, we probed for PPARα, a nuclear receptor that is coactivated by PGC-1 and has been implicated in both mitochondrial biogenesis and fatty acid oxidation [102, 103]. The immunoblots for the mitochondrial biogenesis proteins show that the levels of the proteins are not expressed differently between the wild type and ErbB2 transgenic hearts

(Figure 4.3.A). Our findings suggest that cardiac ErbB2 overexpression does not seem to alter the expression of key mitochondrial biogenesis proteins.

Mitochondrial oxygen consumption and complex I activity are not different between the wild type and ErbB2 transgenic mitochondria

The evidence for the presence of mitochondrial perturbations as a consequence of cardiac ErbB2 overexpression was from a subset of microarray data that indicated a group of complex I genes were downregulated in the ErbB2 transgenic hearts. In order to address whether complex I was dysfunctional in the transgenic hearts, we isolated cardiac mitochondria and performed mitochondrial respiratory function studies using complex I substrates glutamate and malate. The respiratory control ratio (RCR) was calculated as the ratio of state 3 to state 4 respirations and is an important determinant of mitochondrial function and integrity. The data show that the cardiac mitochondria from both groups of animals have similar RCR values; furthermore, the RCR values that we obtained are within the range of RCR values that have been reported for mitochondria isolated from mouse hearts (4.3.B (A)). The amount of oxygen consumed under state 4 (oxygen consumed on complex I substrates) and state 3 respiration (oxygen consumed during the conversion of ADP to ATP) conditions are also similar between the wild type and ErbB2 transgenic mitochondria (4.3.B (B)). Next, we assessed complex I activity and did not find any differences between both groups of animals (4.3.B (C)). We measured respiratory function with succinate, a complex II substrate and observed a lower RCR for the transgenic mitochondria; however, the difference was not statistically significant (4.3.B (D)). The state 3 and state 4 respirations were also not different between the two groups of animals (4.3.B (E)). Collectively, our results indicate that the ErB2 transgenic

cardiac mitochondria are not dysfunctional at the level of complex I, suggesting that cardiac ErbB2 overexpression does not compromise the mitochondria at this region of the electron transport chain.

ATP generated from complex I, II, and IV substrates are not different between the wild type and ErbB2 transgenic mitochondria

One of the most essential functions of the mitochondria is to produce ATP that is necessary for the overall health and survival of the cell. Measurements of ATP generation are typically performed to identify mitochondrial impairments that may be linked to a defective electron transport chain or to oxidative stress. Using isolated cardiac mitochondria, we performed a luminescence assay to determine the amount of ATP in the presence of complex I, II, and IV substrates. As expected, the concentration of ATP that is produced decreases as electrons move progressively down the electron transport chain starting from complex I, as shown in Figure 4.3.C. Furthermore, we did not observe any differences in the amount of ATP between the wild type and ErbB2 transgenic mitochondria.

ErbB2 transgenic mitochondria have a higher baseline mitochondrial membrane potential ($\Delta\varphi$*) than wild type mitochondria*

The $\Delta\varphi$ is an important component of the proton motive force that is necessary for oxidative phosphorylation and ATP generation. Measurements of $\Delta\varphi$ have been utilized as a marker for cellular viability, ROS emission, and mitochondrial indices such as mitochondrial dysfunction and apoptotic signaling[105-107]. For our studies, we quantified the $\Delta\varphi$ using the

TMRE cationic fluorescent dye under nonquenching conditions[108] in the presence of glutamate and malate. Prior to the addition of ADP, the TMRE fluorescence from the ErbB2 transgenic mitochondria at 20 minutes was higher than the fluorescence emitted from the wild type mitochondria (4.3.D). Upon the addition of ADP to stimulate state 3 respiration at 30 minutes, the TMRE fluorescence remained higher in the transgenic mitochondria compared to the wild type mitochondria and persisted until the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was added to completely dissipate the $\Delta\varphi$ (4.3.D). Further support for our findings is from neonatal cardiomyocytes that were stained with the membrane-permeant JC-1 dye that accumulates in polarized mitochondria, resulting in a fluorescent emission from green to red (data not shown). In depolarized mitochondria, however, the JC-1 dye fails to become sequestered into the mitochondria and the red to green fluorescence intensity decreases. Thus, our results indicate that the ErbB2 transgenic mitochondria maintain a higher baseline $\Delta\varphi$ compared to the wild type mitochondria.

ErbB2 transgenic mitochondria have less calcium (Ca^{2+}) uptake compared to the wild type mitochondria

Calcium handling in the cell is intricately linked to mitochondrial processes such as ATP generation and ROS production [105]. For example, mitochondrial Ca^{2+} uptake via the Ca^{2+} uniporter (MCU) is regulated by the $\Delta\varphi$ in order to stimulate the tricarboxylic acid (TCA) cycle, resulting in activation of the electron transport chain and oxidative phosphorylation to ultimately generate ATP [105, 109]. Several hypotheses exist that Ca^{2+} stimulates the production of mitochondrial ROS from various locations of the electron transport chain [105]. However, the exact mechanism for Ca^{2+}-induced mitochondrial ROS

production is still under investigation. For our studies, we measured the Ca^{2+} retention capacity of the mitochondria isolated from the wild type and ErbB2 transgenic hearts. Using the fluorescent probe Calcium Green-5N[110] to measure extramitochondrial Ca^{2+}, we determined that the ErbB2 transgenic mitochondria require less pulses of Ca^{2+} compared to the wild type mitochondria in order to reach a point of saturation, whereby the fluorescent intensity is at a maximum (4.3.E). The representative curves that are displayed in Figure 4.3.E show that over a period of one hour the wild type mitochondria have a greater capacity to uptake and release Ca^{2+} compared to the ErbB2 transgenic mitochondria. Our results suggest that the ErbB2 transgenic mitochondria have less mitochondrial Ca^{2+} uptake due to altered mechanisms for Ca^{2+} entry, such as through the MCU or the other modes of $Ca2^{+}$ uptake that consists of the rapid-mode Ca^{2+} uptake (RaM) and the ryanodine receptor (RyR) [105, 109]. Another plausible explanation is that the ErbB2 mitochondria are in a swollen state thereby preventing Ca^{2+} from either entering or exiting the mitochondria.

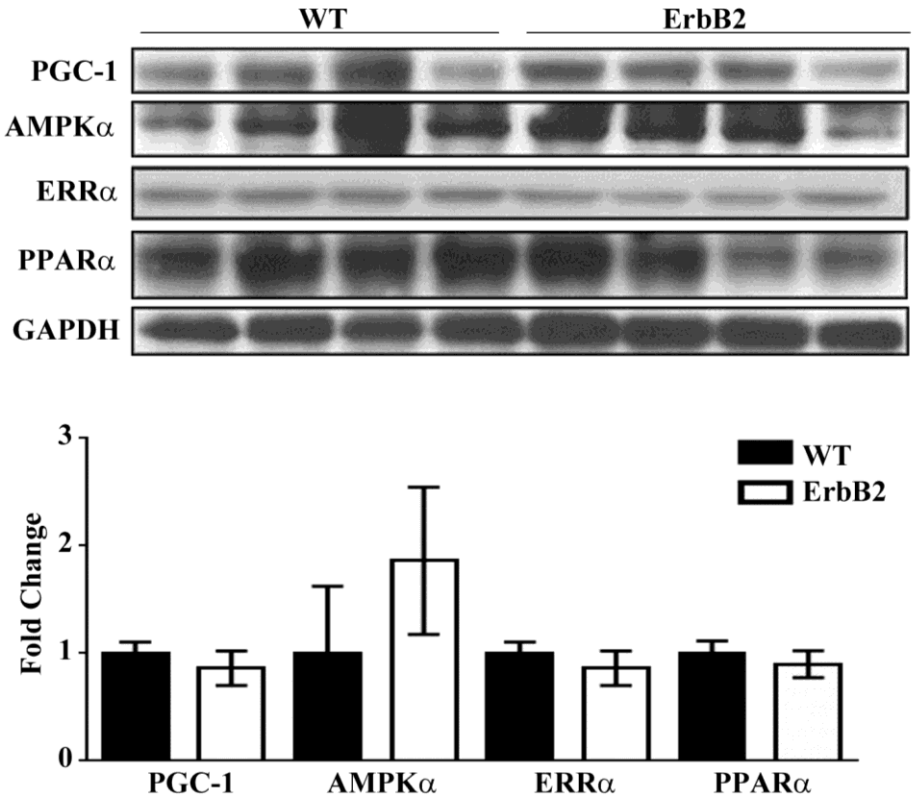


Fig. 4-1. Expression of key mitochondrial biogenesis proteins. Immunoblots show that the levels of various mitochondrial biogenesis proteins are not different between the wild type and ErbB2 transgenic mice (n=4 per group). Values represent the means and SD.

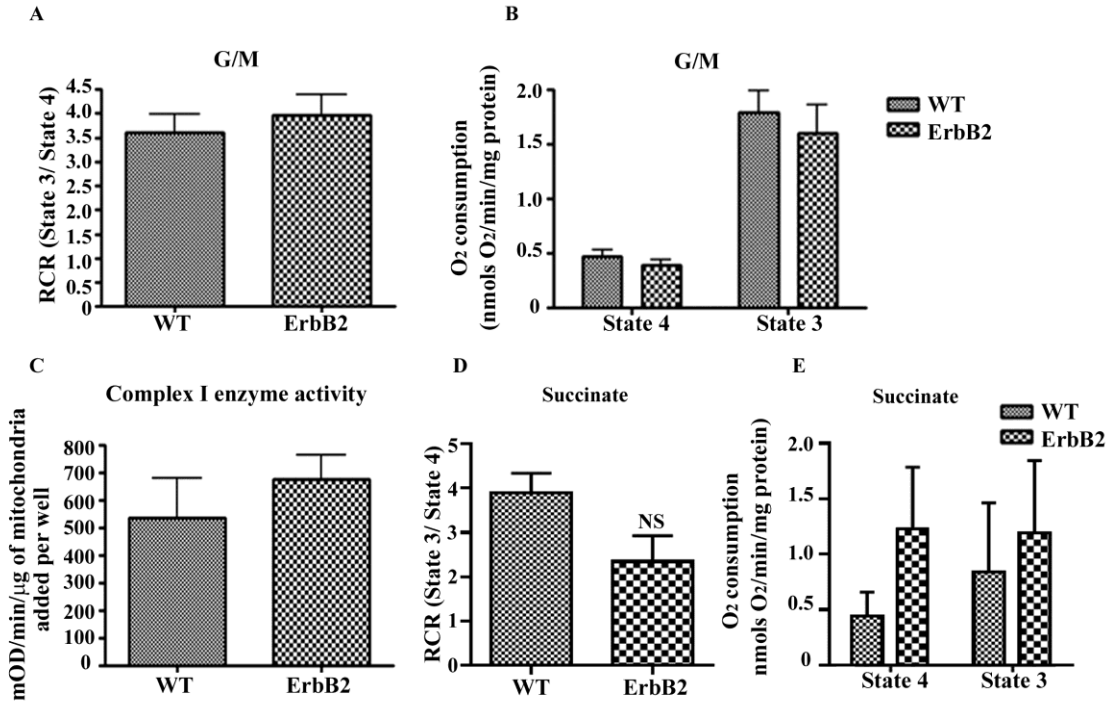


Fig. 4-2. Mitochondrial respiration studies with substrates for complex I and II indicate that respiratory function of isolated mitochondria from the ErbB2 transgenic hearts is not different compared to wild type. (A) The respiratory control ratio (RCR; state 3/state 4) is an index of the integrity of the isolated mitochondria; results show that the RCR (B) state 4 and state 3 respiratory rates (n=8 per group) and (C) complex I enzyme activity are similar between both groups of animals (n=6 per group). (D) Respiratory assays with the complex II substrate succinate indicate that the (D) RCR and (E) state 3 and state 4 respiratory rates are not different between both groups of animals (n=3 per group). Values represent the means and SD.

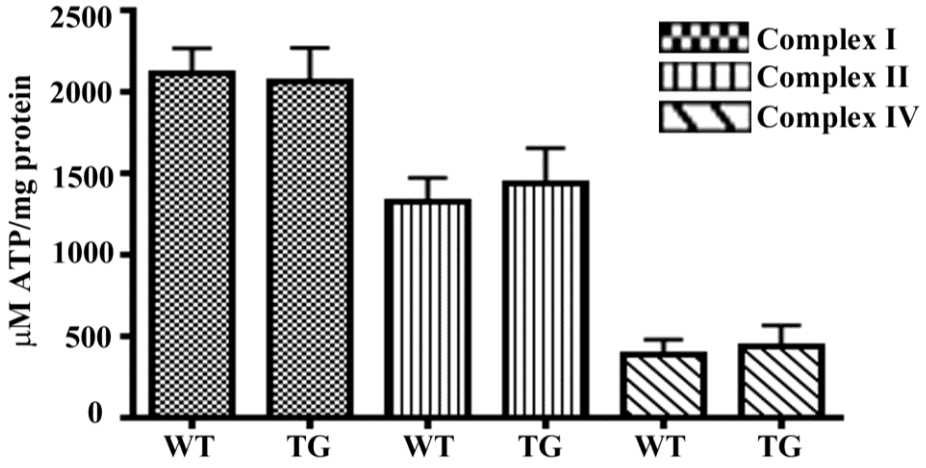
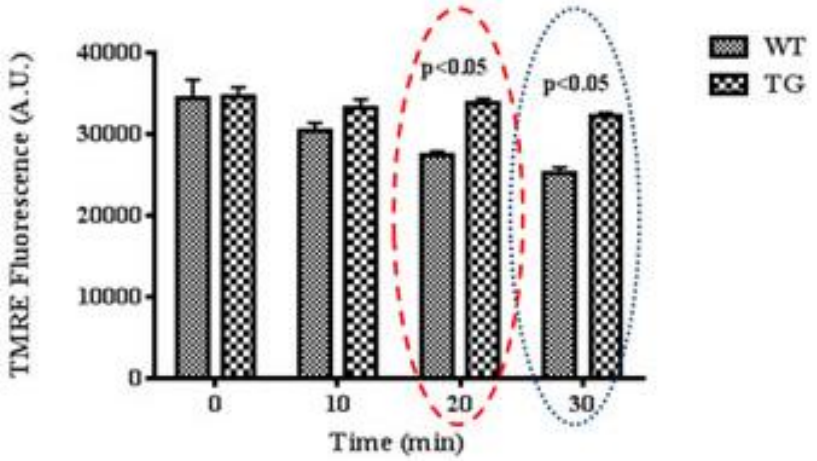


Fig. 4-3. ATP production from isolated heart mitochondria is similar between both groups of animals. The decrease in ATP production with substrates for complex I, II, and IV is an expected trend (n=6 per group). Values represent the means and SD.

Fig. 4-4. Mitochondria isolated from the ErbB2^{tg} hearts have a higher mitochondrial membrane potential (Δφ) compared to wild type heart mitochondria. The Δφ was assessed in a nonquenching mode whereby depolarized mitochondria accumulate less of the cationic Tetramethylrhodamine, Ethyl Ester, Perchlorate (TMRE) dye compared to hyperpolarized mitochondria that will have a higher TMRE fluorescence due to a greater concentration of TMRE in the mitochondria. The Δφ for each group was quantified based on the average TMRE fluorescence that is represented by the curves in the bottom graph (n=3 per group). The bar graph (top) was generated from the corresponding TMRE fluorescent values that are shown in the curves. After 20 and 30 minutes the Δφ was higher in the ErbB2^{tg} heart mitochondria compared to mitochondria isolated from the wild type hearts. *$p<0.05$. Values represent the means and SD.


TMRE Fluorescence (A.U.)
40000
30000
20000
10000
0
p<0.05
p<0.05
WT
TG
0
10
20
30
Time (min)

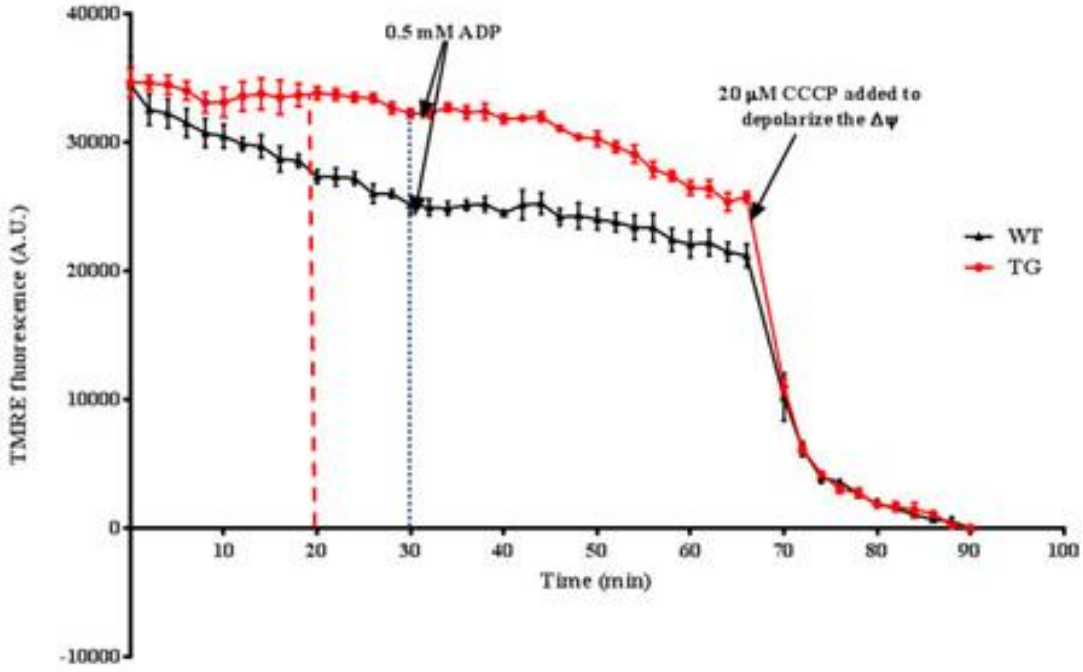
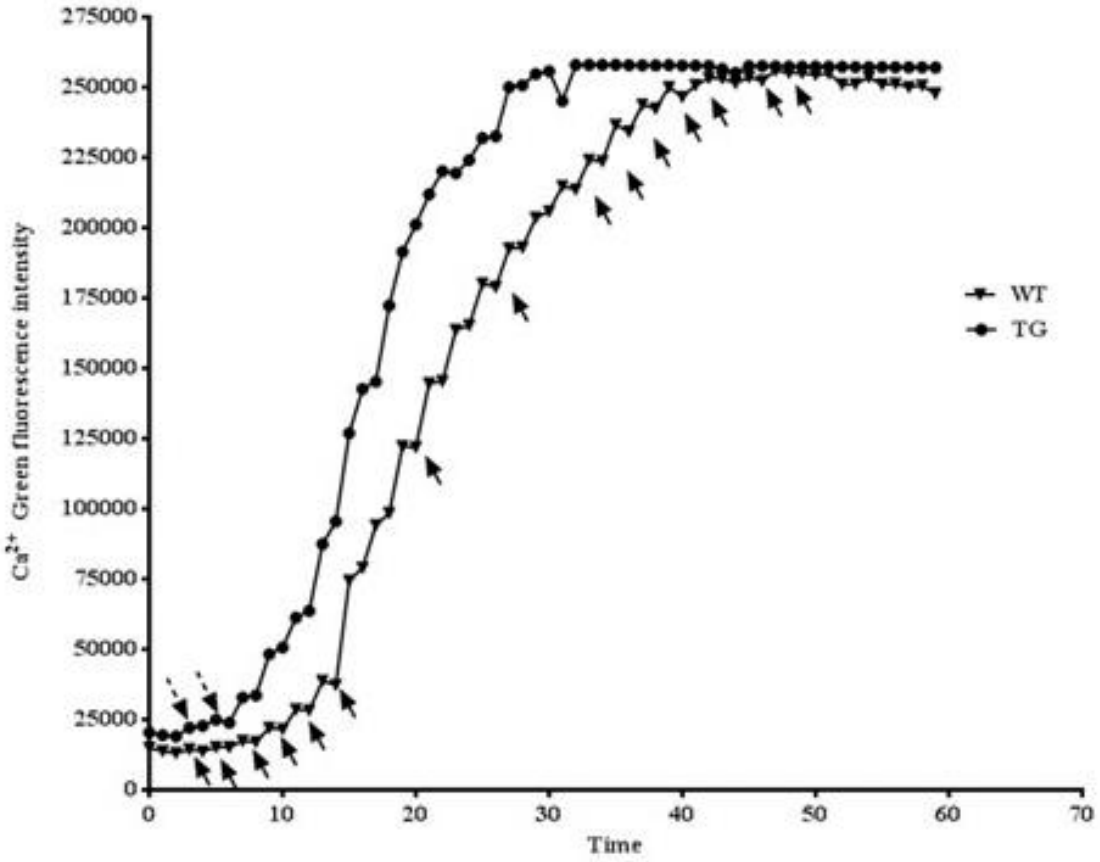
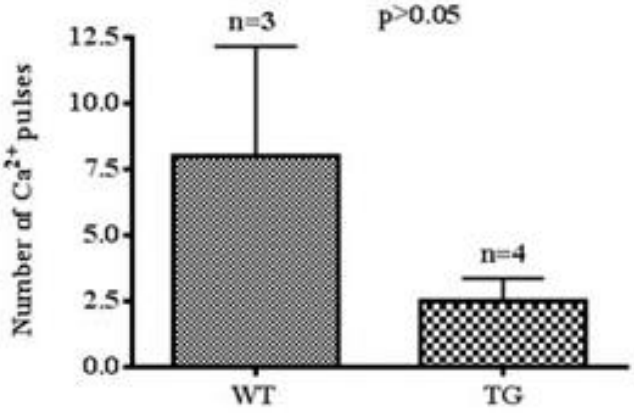

TMRE fluorescence (A.U.)
40000
30000
20000
10000
0
-10000
0.5 mM ADP
20 µM CCCP added to depolarize the Δψ
WT
TG
10
20
30
40
50
60
70
80
90
100
Time (min)

Fig. 4-5. The ErbB2^{tg} mitochondria have less Ca^{2+} uptake compared to wild type mitochondria. Representative curves of the wild type and ErbB2^{tg} mitochondria are shown in the top graph, which indicates that the wild type mitochondria require more pulses of Ca^{2+} as indicated by the arrows. The number of Ca^{2+} pulses quantified for each group is shown in the bottom graph. *$p<0.05$. Values represent the means and SD.



Calcium Retention



4.4 Discussion

The high demand that is imposed upon the heart to produce ATP through oxidative metabolism necessitates the large density of mitochondria that are contained in cardiac myocytes. Moreover, the importance of cardiac mitochondria in heart function requires that these organelles have the ability to adapt to changes in the energy requirements of the cell. In the case of cardiac hypertrophy that is induced by increased workload such as during exercise, the mitochondria respond to the energetic demands by proliferating and producing sufficient amounts of ATP [98]. If, however, the hypertrophy becomes decompensated, mitochondrial dysfunction ensues that may ultimately lead to heart failure [98]. In our model of cardiac ErbB2 overexpression we observed a striking hypertrophic phenotype without progression to heart failure. Our findings coupled with microarray data to suggest that mitochondrial complex I genes were downregulated led us to further investigate the effects from ErbB2 overexpression on the mitochondria. We probed for proteins that are critical for mitochondrial biogenesis and can be altered in hypertrophy. Our results indicate that the expression of PGC-1, AMPKα, ERRα, and PPARα proteins is not different between the wild type and ErbB2 transgenic hearts. Thus, cardiac ErbB2 overexpression and the resulting hypertrophy do not perturb the protein levels of key mitochondrial biogenesis factors. Further support for our findings is from electron microscopy images of ErbB2 transgenic mitochondria that appear to have normal morphology (data not shown).

Rather than validate the microarray data via qPCR we opted instead to examine complex I and II respiratory function and complex I activity. Our results show that there are no differences in the RCR or state 3 and state 4 respiration with complex I and II

substrates as well as in complex I activity between both groups of animals. Possible explanations for the discrepancies between the microarray data and our mitochondrial respiration studies are that the downregulated genes do not affect the translation or degradation of the proteins that are necessary for mitochondrial function. Another plausible explanation is that the ErbB2 transgenic mitochondria are equipped with compensatory mechanisms that can sufficiently overcome any mitochondrial deficits. Although we did not observe any mitochondrial dysfunction at the level of complex I and II, it is possible that mitochondrial impairments may exist further downstream of the electron transport chain. Additional studies are necessary to validate the microarray data and to more accurately assess mitochondrial function with different mitochondrial respiratory chain substrates.

A known consequence of mitochondrial dysfunction and hypertrophy is impaired ATP production[98, 111]. We determined that the ErbB2 transgenic mitochondria do not produce less ATP with complex I, II, and IV substrates compared to wild type mitochondria. Reduced ATP production occurs during decompensated, pathological hypertrophy that is a contributing factor for the onset of heart failure [98]. Thus, our findings provide additional insight on whether ErbB2 overexpression induces a physiological or compensated form of hypertrophy.

One of the most interesting findings from the mitochondrial characterization studies were from the $\Delta\varphi$ assays where we showed through two different methods—TMRE fluorescence and JC-1 staining of neonatal mouse cardiomyocytes—that the ErbB2 transgenic hearts have a higher $\Delta\varphi$ compared to wild type hearts. Since the $\Delta\varphi$ is an important indicator of cellular viability, the higher $\Delta\varphi$ in the transgenic hearts supports

our previous findings that the pro-survival, anti-apoptotic proteins Bcl2 and Bcl-xL are increased in the transgenic hearts. For example, it has been hypothesized that an increase in $\Delta\varphi$ may serve as an adaptive response against apoptosis, particularly in the transition from compensated hypertrophy to heart failure [112]. While our $\Delta\varphi$ data support the anti-apoptotic phenotype from cardiac ErbB2 overexpression, the high $\Delta\varphi$ suggests that the transgenic hearts would have more ROS production and oxidative stress rather than less, which we have shown through three different methods in the previous chapter. A possible explanation for these disparate results is that the antioxidant capacity in the transgenic hearts is elevated under hypertrophy as a protective mechanism against oxidative stress. The distinct role of $\Delta\varphi$ on ROS regulation warrants further examination, as other groups have found that a depolarized $\Delta\varphi$ was associated with an increase in ROS production [113].

The integral role of Ca^{2+} in mitochondrial function is to stimulate oxidative phosphorylation for ATP synthesis. When Ca^{2+} homeostasis is disrupted, such as in pathological hypertrophy, mitochondrial dysfunction ensues that may accompanied by increased ROS and cellular death [105, 114].We measured mitochondrial Ca^{2+} handling and found that the ErbB2 transgenic mitochondria have less Ca^{2+} uptake compared to the wild type mitochondria. The precise mechanism for cardiac ErbB2 overexpression on mitochondrial Ca^{2+} in our transgenic model is poorly understood. Adding to the complexity of our mitochondrial Ca^{2+} findings are studies that link mitochondrial matrix Ca^{2+} ($[Ca^{2+}]_m$) overload to increased ROS and opening of the permeability transition pore resulting in apoptosis[115 105]. However, we observed less ROS and apoptosis in the ErbB2 transgenic hearts; further studies are needed to explain the discordancy in our findings. Since Ca^{2+} stimulates the TCA cycle, we cannot rule out the possibility that the reduced

uptake of Ca^{2+} by the ErbB2 transgenic mitochondria is somehow related to TCA cycle activation [114]. Thus, for future studies, we will measure $[Ca^{2+}]_m$ and α-ketoglutarate dehydrogenase and pyruvate dehydrogenase enzymatic activities to assess whether $[Ca^{2+}]_m$ in the ErbB2 transgenic mitochondria is increasing the activity of these enzymes. Lastly, we observed in separate studies that the ErbB2 transgenic hearts have higher baseline Ca^{2+} transients and faster Ca^{2+} reuptake. However, additional studies are needed to understand how ErbB2 overexpression regulates Ca^{2+} in the cardiomyocyte, particularly in the cytosol and mitochondria.

In summary, our mitochondrial characterization studies provided much-needed insight on whether cardiac ErbB2 overexpression and the resulting hypertrophy lead to mitochondrial alterations that are characteristic of physiological and pathological hypertrophy. Based on our findings, we were able to determine that ErbB2 overexpression does not affect key mitochondrial biogenesis proteins or respiratory function with complex I and II substrates. Furthermore, while we did not observe deficits in ATP production, we found that the $\Delta\varphi$ is increased and that the transgenic mitochondria have less Ca^{2+} uptake. Although the exact mechanism for ErbB2-induced hypertrophy on the $\Delta\varphi$ and $[Ca^{2+}]_m$ are unknown, our studies may prove useful for understanding mitochondrial processes that are regulated by tyrosine kinase signaling and hypertrophy, particularly in cases where there is an absence of heart failure.

4.5 Methods and Materials

Animals

The animal protocol was followed as described in the preceding chapter, section 3.5.

Immunoblotting

The immunblotting protocol that was described in section 3.5 of the previous chapter was used but with slight modifications. The primary antibodies used were directed against PGC-1 (Santa Cruz, Santa Cruz, CA), AMPKα (cell Signaling, Danvers, MA), ERRα, and PPARα (Millipore, Billerica, MA). Levels of GAPDH (Santa Cruz, Santa Cruz, CA) protein were measured to normalize protein quantities across samples.

Mitochondrial Isolation from Hearts

The protocol for isolating mitochondria from heart tissue was followed as described in section 3.5 of the preceding chapter.

Mitochondrial Respiration Studies

Respiration was measured using freshly isolated heart mitochondria (200µg of mitochondrial protein resuspended in 10mM Hepes, 0.25M Sucrose, 1mM EGTA, 0.1% BSA) that was added to a 25°C chamber containing mitochondrial respiration buffer (120mM KCl, 1mM EGTA, 5mM MOPS, 5mM K_2HPO_4, 0.2% BSA). Oxygen consumption was monitored with a Clark-type O_2 electrode (Instech, Plymouth Meeting, PA) connected to an O_2 monitor (Model 5300, YSI, Inc., Yellow Springs, OH)[116, 117]. Glutamate/malate (10mM/2mM) or succinate (5mM) was added to the chamber followed by ADP (0.5mM) for state 3 respiration. After the ADP was converted to ATP, the state 4 respiratory rate was measured.

ATP Measurements

Isolated heart mitochondria were placed in mitochondrial respiration buffer (120mM KCl, 1mM EGTA, 5mM MOPS, 5mM K_2HPO_4, 0.2% BSA) and incubated with glutamate/malate (10mM/2mM), succinate (5mM), or TMPD/ascorbate (0.2mM/5mM)

with ADP (0.5mM), oligomycin (1μg/mL), and 2,4-dinitrophenol (DNP, 50μM). The samples were centrifuged and the supernatant was collected for ATP measurements using the luminescence-based ATP Determination Kit (Molecular Probes, Eugene, OR). Briefly, a reaction buffer and stock solutions for D-luciferin and dithiothreitol (DTT) were prepared according to the manufacturer's instructions; a standard reaction solution was prepared by combining the components along with firefly luciferase. ATP standard solutions were also prepared to generate a standard curve. The standard reaction solution was dispensed into each sample well and the luminescence was recorded.

Measurements of mitochondrial membrane potential (Δφ)

The Δφ was measured using a 96-well fluorescent plate reader and 10nM of tetramethylrhodamine ethyl ester (TMRE) that was added to the isolated heart mitochondria (350μg mitochondrial protein/well) contained in the appropriate buffer (135mM KCl, 20 mM MOPS, 5 mM K_2HPO_4, 5mM $MgCl_2$, pH 7.0)[116]. The respiratory substrates glutamate/malate (10mM/2mM) were added to the mitochondria at the beginning of the assay. After approximately 30min ADP (0.5mM) was added to allow state 3 respiration to occur. Thirty minutes following the addition of ADP, the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, 20μM) was added to dissipate the Δφ. Fluorescent readings were recorded at an excitation of 544nm and 590nm emission.

Mitochondrial calcium (Ca^{2+}) retention capacity

A 96-well fluorescent plate reader and the Calcium Green 5N fluorescent indicator[110] (2μM, Life Technologies, Green Island, NY) were used to measure extramitochondrial Ca^{2+}. The mitochondria (300μg) were contained in mitochondrial swelling buffer (120mM KCl, 10mM tris-HCl, 5mM MOPS, 5mM KH_2PO_4, 0.005mM EGTA) and

glutamate/malate (10mM/2mM) was used as the respiratory substrates. A stock of $CaCl_2$ (0.5mM) was prepared in ddH_2O and was added for a final concentration of 20µM to each well every minute up to 60 min. The fluorescence was recorded at an excitation of 485nm and 529nm emission.

Chapter 5

Conclusions and additional studies

5.1 Chronic overexpression of ErbB2 in the heart as a model to study ROS and mitochondrial function

The work that is presented in this dissertation adds to a growing body of research that ErbB2 serves a critical role in the heart. Using the ErbB2^{tg} mouse model we provide evidence for a novel redox signaling pathway regulated by ErbB2 overexpression in the heart, whereby Abl kinases are upregulated to activate Gpx in order to reduce ROS production. The heightened antioxidant capacity in the ErbB2^{tg} hearts is a likely factor (among many that have yet to be discovered) involved in the reduced propensity of the hypertrophic transgenic hearts from going into heart failure. Additional studies will be performed with neonatal cardiomyocytes isolated from wild type and ErbB2^{tg} hearts to confirm our *in vivo* findings that ErbB2 regulates GPx, particularly under hypertrophy.

The mitochondrial studies will serve as a foundation for future research into how ErbB2 uniquely regulates mitochondrial function. As we have seen in our work, both the $\Delta\varphi$ and Ca^{2+} handling are significantly different compared to wild type heart mitochondria. In fact, very preliminary studies that employed sensitive, cuvette-based fluorometric techniques confirmed that the $\Delta\varphi$ is higher and ROS levels are lower in the ErbB2^{tg} heart mitochondria. However, these assays revealed that ubiquinone of complex I may be damaged in the wild type mitochondria, suggesting that the mitochondrial impairments are strain-specific or that ErbB2 overexpression is activating compensatory mechanisms against these mitochondrial deficits. More studies are necessary to carefully investigate these findings.

The ErbB2^{tg} model continues to offer new and interesting avenues for research into signaling pathways that are regulated by ErbB2 overexpression. Though much work

is needed to fully understand the complexities of ErbB2 overexpression in the heart, our work uncovers novel mechanisms for the protective effects of ErbB2 in heart. Finally, the translational significance of these studies will be for the development of drugs that are efficacious in cancer therapy but are designed to exclude cardiotoxic effects.

5.2 Additional studies

5.2.1 Establishing an in vitro model of ErbB2 overexpression in HEK293 and H9c2 cells

The plasmids for the pcDNA 3.1(+) vector and R-neu were designed by Dr. Yi Xu. The following data are from studies that were performed in order to establish an *in vitro* model of ErbB2 overexpression in HEK293 and H9c2 cell lines. Transfection protocols were optimized to ensure that the plasmid delivery and transfection efficiency (determined via immunblotting) were optimal.

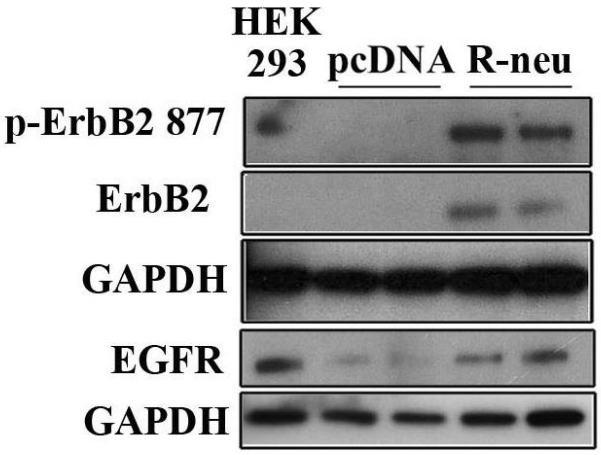


Fig. 5-1. HEK293 cells transiently transfected with the control plasmid (pcDNA) or ErbB2-overexpressing plasmid (R-neu). Immunoblotting results show that R-neu-transfected cells express more ErbB2 compared to vector-transfected cells, with a concurrent upregulation of phosphorylated ErbB2 (p-ErbB2 877) and EGFR proteins. GAPDH was used as a loading control.

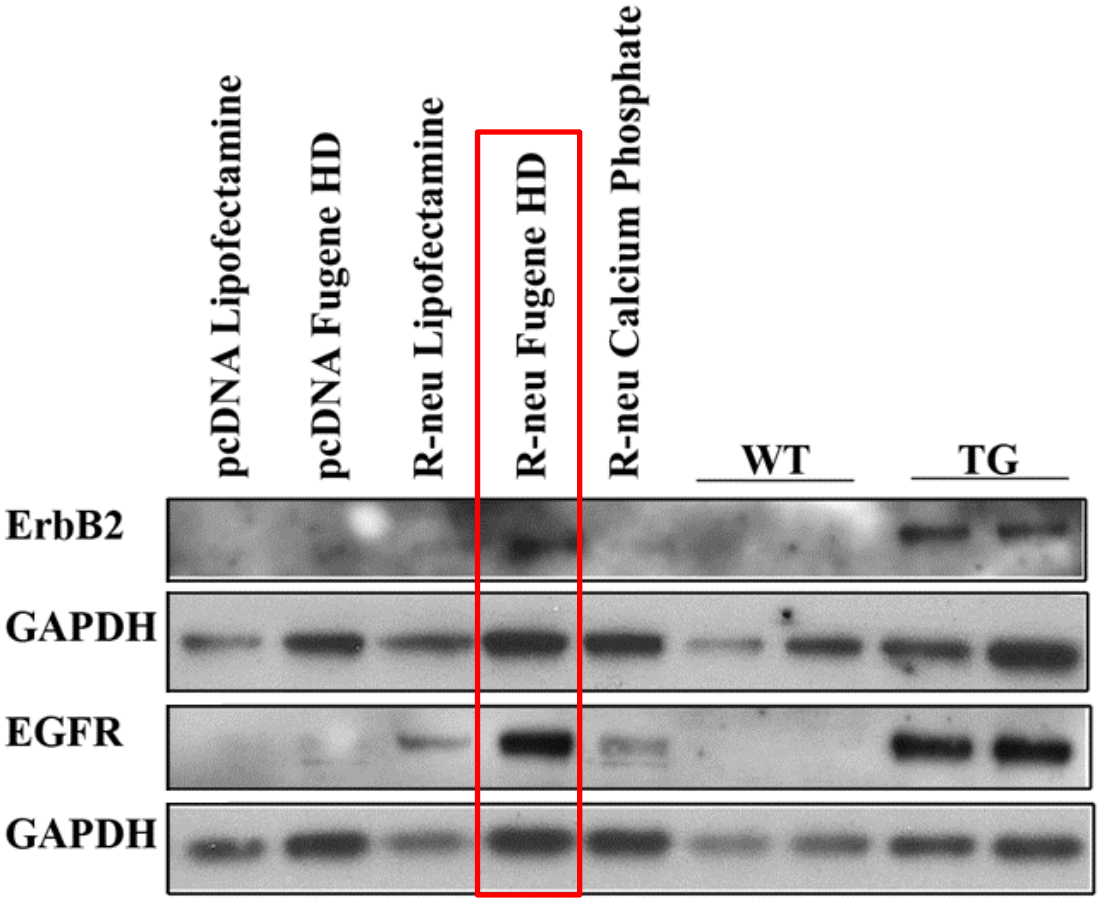


Fig. 5-2. The H9c2 rat cardiomyoblast cell line was selected for subsequent *in vitro* studies of ErbB2 overexpression and knockdown. Shown here are immunoblots of ErbB2 and EGFR proteins based on three different transfection protocols. Four our studies, the transfection reagent Fugene HD caused the least amount of cellular death and provided a strong overexpression of ErbB2 protein compared to the other transfection methods.

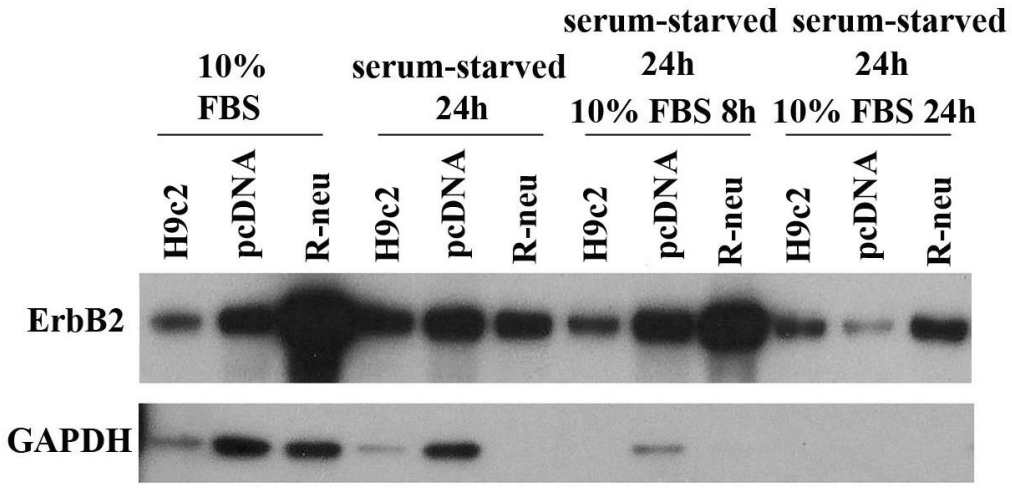


Fig. 5-3. Serum (10% FBS) starvation of transfected H9c2 cells. A series of experiments were performed to assess whether serum starvation was necessary for the H9c2 transfections. We determined that serum starvation was not needed for transfections. The variability in GAPDH protein expression may be a consequence of the serum conditions.

5.2.2 Overexpression of ErbB2 in HEK293 cells upregulates β2-adrenergic receptor (β2-AR)

Recently submitted work that investigated the role of cardiac ErbB2 in β2-AR signaling included the following figure that shows immunoblots for ErB2 overexpression in HEK293 cells. Most importantly, however, is that ErbB2 overexpression upregulates β2-AR, which is a finding that was confirmed in the ErbB2^{tg} hearts.

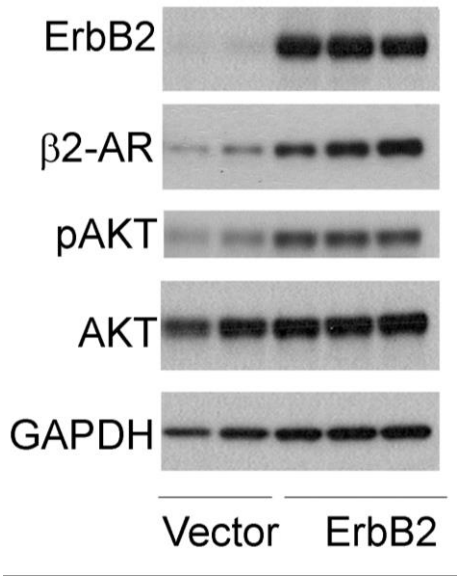


Fig. 5-4. HEK293 cells transfected with ErbB2 plasmid have a concurrent upregulation of β2-AR and phosphorylated AKT proteins.

5.2.3 Acute treatment with doxorubicin reduces the survival of ErbB2^{tg} mice

In separate studies with doxorubicin, wild type or ErbB2^{tg} mice were administered a single dose of 20 mg/kg doxorubicin by intraperitoneal injection. Control animals received an equivalent volume of saline. Mortality was assessed over a four-day period after the injections and the surviving animals were euthanized after four days. Our results indicate that the ErbB2^{tg} mice have a lower tolerance for doxorubicin, as indicated by the reduced survival of the ErbB2^{tg} mice compared to wild type-treated animals.

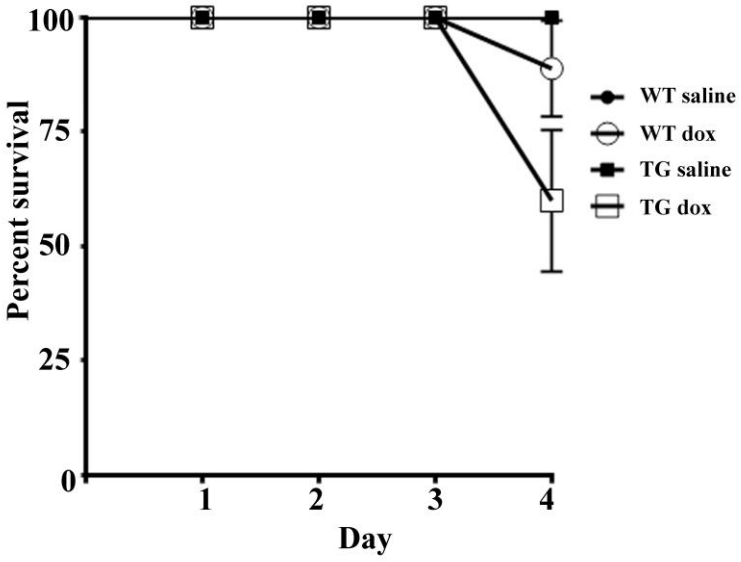


Fig. 5-5. Doxorubicin treatment and survival. Acute treatment with doxorubicin (dox) significantly reduced the survival of the transgenic (TG) mice compared to similarly treated wild type animals (n=6 per group). For survival statistics Log-rank (Mantel-Cox) test was used.

References

1. Cohen S. The stimulation of epidermal proliferation by a specific protein (egf). *Dev Biol*. 1965;12:394-407
2. Gschwind A, Fischer OM, Ullrich A. The discovery of receptor tyrosine kinases: Targets for cancer therapy. *Nat Rev Cancer*. 2004;4:361-370
3. Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD. Close similarity of epidermal growth factor receptor and v-erb-b oncogene protein sequences. *Nature*. 1984;307:521-527
4. Burden S, Yarden Y. Neuregulins and their receptors: A versatile signaling module in organogenesis and oncogenesis. *Neuron*. 1997;18:847-855
5. Yarden Y, Sliwkowski MX. Untangling the erbb signalling network. *Nat Rev Mol Cell Biol*. 2001;2:127-137
6. Klapper LN, Glathe S, Vaisman N, Hynes NE, Andrews GC, Sela M, Yarden Y. The erbb-2/her2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors. *Proc Natl Acad Sci U S A*. 1999;96:4995-5000
7. Graus-Porta D, Beerli RR, Daly JM, Hynes NE. Erbb-2, the preferred heterodimerization partner of all erbb receptors, is a mediator of lateral signaling. *EMBO J*. 1997;16:1647-1655
8. Tzahar E, Waterman H, Chen X, Levkowitz G, Karunagaran D, Lavi S, Ratzkin BJ, Yarden Y. A hierarchical network of interreceptor interactions determines signal transduction by neu differentiation factor/neuregulin and epidermal growth factor. *Mol Cell Biol*. 1996;16:5276-5287

9. Pinkas-Kramarski R, Soussan L, Waterman H, Levkowitz G, Alroy I, Klapper L, Lavi S, Seger R, Ratzkin BJ, Sela M, Yarden Y. Diversification of neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J*. 1996;15:2452-2467

10. Ding Y, Liu Z, Desai S, Zhao Y, Liu H, Pannell LK, Yi H, Wright ER, Owen LB, Dean-Colomb W, Fodstad O, Lu J, LeDoux SP, Wilson GL, Tan M. Receptor tyrosine kinase erbb2 translocates into mitochondria and regulates cellular metabolism. *Nat Commun*. 2012;3:1271

11. Codony-Servat J, Albanell J, Lopez-Talavera JC, Arribas J, Baselga J. Cleavage of the her2 ectodomain is a pervanadate-activable process that is inhibited by the tissue inhibitor of metalloproteases-1 in breast cancer cells. *Cancer Res*. 1999;59:1196-1201

12. Pupa SM, Menard S, Morelli D, Pozzi B, De Palo G, Colnaghi MI. The extracellular domain of the c-erbb-2 oncoprotein is released from tumor cells by proteolytic cleavage. *Oncogene*. 1993;8:2917-2923

13. Lin YZ, Clinton GM. A soluble protein related to the her-2 proto-oncogene product is released from human breast carcinoma cells. *Oncogene*. 1991;6:639-643

14. Zabrecky JR, Lam T, McKenzie SJ, Carney W. The extracellular domain of p185/neu is released from the surface of human breast carcinoma cells, sk-br-3. *J Biol Chem*. 1991;266:1716-1720

15. Tan M, Li P, Klos KS, Lu J, Lan KH, Nagata Y, Fang D, Jing T, Yu D. Erbb2 promotes src synthesis and stability: Novel mechanisms of src activation that confer breast cancer metastasis. *Cancer Res*. 2005;65:1858-1867

16. Tan M, Yao J, Yu D. Overexpression of the c-erbb-2 gene enhanced intrinsic metastasis potential in human breast cancer cells without increasing their transformation abilities. *Cancer Res*. 1997;57:1199-1205

17. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci U S A*. 1992;89:10578-10582

18. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, et al. Studies of the her-2/neu proto-oncogene in human breast and ovarian cancer. *Science*. 1989;244:707-712

19. Ross JS, Fletcher JA. The her-2/neu oncogene in breast cancer: Prognostic factor, predictive factor, and target for therapy. *Oncologist*. 1998;3:237-252

20. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: Correlation of relapse and survival with amplification of the her-2/neu oncogene. *Science*. 1987;235:177-182

21. Lee KF, Simon H, Chen H, Bates B, Hung MC, Hauser C. Requirement for neuregulin receptor erbb2 in neural and cardiac development. *Nature*. 1995;378:394-398

22. Gassmann M, Casagranda F, Orioli D, Simon H, Lai C, Klein R, Lemke G. Aberrant neural and cardiac development in mice lacking the erbb4 neuregulin receptor. *Nature*. 1995;378:390-394

23. Meyer D, Birchmeier C. Multiple essential functions of neuregulin in development. *Nature*. 1995;378:386-390

24. Sawyer DB, Caggiano A. Neuregulin-1beta for the treatment of systolic heart failure. *J Mol Cell Cardiol*. 2011;51:501-505

25. Ozcelik C, Erdmann B, Pilz B, Wettschureck N, Britsch S, Hubner N, Chien KR, Birchmeier C, Garratt AN. Conditional mutation of the erbb2 (her2) receptor in cardiomyocytes leads to dilated cardiomyopathy. *Proc Natl Acad Sci U S A*. 2002;99:8880-8885

26. Crone SA, Zhao YY, Fan L, Gu Y, Minamisawa S, Liu Y, Peterson KL, Chen J, Kahn R, Condorelli G, Ross J, Jr., Chien KR, Lee KF. Erbb2 is essential in the prevention of dilated cardiomyopathy. *Nat Med*. 2002;8:459-465

27. Makki N, Thiel KW, Miller FJ, Jr. The epidermal growth factor receptor and its ligands in cardiovascular disease. *Int J Mol Sci*. 2013;14:20597-20613

28. Gordon LI, Burke MA, Singh AT, Prachand S, Lieberman ED, Sun L, Naik TJ, Prasad SV, Ardehali H. Blockade of the erbb2 receptor induces cardiomyocyte death through mitochondrial and reactive oxygen species-dependent pathways. *J Biol Chem*. 2009;284:2080-2087

29. Singh KK, Shukla PC, Quan A, Lovren F, Pan Y, Wolfstadt JI, Gupta M, Al-Omran M, Leong-Poi H, Teoh H, Verma S. Herceptin, a recombinant humanized anti-erbb2 monoclonal antibody, induces cardiomyocyte death. *Biochem Biophys Res Commun*. 2011;411:421-426

30. Grazette LP, Boecker W, Matsui T, Semigran M, Force TL, Hajjar RJ, Rosenzweig A. Inhibition of erbb2 causes mitochondrial dysfunction in

cardiomyocytes: Implications for herceptin-induced cardiomyopathy. *J Am Coll Cardiol*. 2004;44:2231-2238

31. Pugatsch T, Abedat S, Lotan C, Beeri R. Anti-erbb2 treatment induces cardiotoxicity by interfering with cell survival pathways. *Breast Cancer Res*. 2006;8:R35

32. Rohrbach S, Niemann B, Silber RE, Holtz J. Neuregulin receptors erbb2 and erbb4 in failing human myocardium -- depressed expression and attenuated activation. *Basic Res Cardiol*. 2005;100:240-249

33. Sysa-Shah P, Xu Y, Guo X, Belmonte F, Kang B, Bedja D, Pin S, Tsuchiya N, Gabrielson K. Cardiac-specific over-expression of epidermal growth factor receptor 2 (erbb2) induces pro-survival pathways and hypertrophic cardiomyopathy in mice. *PLoS One*. 2012;7:e42805

34. Giraud MN, Fluck M, Zuppinger C, Suter TM. Expressional reprogramming of survival pathways in rat cardiocytes by neuregulin-1beta. *J Appl Physiol (1985)*. 2005;99:313-322

35. Timolati F, Ott D, Pentassuglia L, Giraud MN, Perriard JC, Suter TM, Zuppinger C. Neuregulin-1 beta attenuates doxorubicin-induced alterations of excitation-contraction coupling and reduces oxidative stress in adult rat cardiomyocytes. *J Mol Cell Cardiol*. 2006;41:845-854

36. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L. Use of chemotherapy plus a monoclonal antibody against her2 for metastatic breast cancer that overexpresses her2. *N Engl J Med*. 2001;344:783-792

37. Suter TM, Cook-Bruns N, Barton C. Cardiotoxicity associated with trastuzumab (herceptin) therapy in the treatment of metastatic breast cancer. *Breast*. 2004;13:173-183

38. Behr TM, Behe M, Wormann B. Trastuzumab and breast cancer. *N Engl J Med*. 2001;345:995-996

39. Liu FF, Stone JR, Schuldt AJ, Okoshi K, Okoshi MP, Nakayama M, Ho KK, Manning WJ, Marchionni MA, Lorell BH, Morgan JP, Yan X. Heterozygous knockout of neuregulin-1 gene in mice exacerbates doxorubicin-induced heart failure. *Am J Physiol Heart Circ Physiol*. 2005;289:H660-666

40. Gabrielson K, Bedja D, Pin S, Tsao A, Gama L, Yuan B, Muratore N. Heat shock protein 90 and erbb2 in the cardiac response to doxorubicin injury. *Cancer Res*. 2007;67:1436-1441

41. Belsches-Jablonski AP, Biscardi JS, Peavy DR, Tice DA, Romney DA, Parsons SJ. Src family kinases and her2 interactions in human breast cancer cell growth and survival. *Oncogene*. 2001;20:1465-1475

42. Dhalla AK, Hill MF, Singal PK. Role of oxidative stress in transition of hypertrophy to heart failure. *J Am Coll Cardiol*. 1996;28:506-514

43. Takimoto E, Kass DA. Role of oxidative stress in cardiac hypertrophy and remodeling. *Hypertension*. 2007;49:241-248

44. Kirshenbaum LA, Hill M, Singal PK. Endogenous antioxidants in isolated hypertrophied cardiac myocytes and hypoxia-reoxygenation injury. *J Mol Cell Cardiol*. 1995;27:263-272

45. Gupta M, Singal PK. Higher antioxidative capacity during a chronic stable heart hypertrophy. *Circ Res*. 1989;64:398-406

46. Dhalla AK, Singal PK. Antioxidant changes in hypertrophied and failing guinea pig hearts. *Am J Physiol*. 1994;266:H1280-1285

47. Duval C, Auge N, Frisach MF, Casteilla L, Salvayre R, Negre-Salvayre A. Mitochondrial oxidative stress is modulated by oleic acid via an epidermal growth factor receptor-dependent activation of glutathione peroxidase. *Biochem J*. 2002;367:889-894

48. Cheadle C, Vawter MP, Freed WJ, Becker KG. Analysis of microarray data using z score transformation. *J Mol Diagn*. 2003;5:73-81

49. Giraud MN, Fluck M, Zuppinger C, Suter TM. Expressional reprogramming of survival pathways in rat cardiocytes by neuregulin-1beta. *J Appl Physiol*. 2005;99:313-322

50. Kowaltowski AJ, de Souza-Pinto NC, Castilho RF, Vercesi AE. Mitochondria and reactive oxygen species. *Free Radic Biol Med*. 2009;47:333-343

51. Miura T, Tanno M, Sato T. Mitochondrial kinase signalling pathways in myocardial protection from ischaemia/reperfusion-induced necrosis. *Cardiovasc Res*. 2010;88:7-15

52. Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J*. 2009;417:1-13

53. Cao C, Leng Y, Huang W, Liu X, Kufe D. Glutathione peroxidase 1 is regulated by the c-abl and arg tyrosine kinases. *J Biol Chem*. 2003;278:39609-39614

54. Srinivasan D, Kaetzel DM, Plattner R. Reciprocal regulation of abl and receptor tyrosine kinases. *Cell Signal*. 2009;21:1143-1150

55. Srinivasan D, Plattner R. Activation of abl tyrosine kinases promotes invasion of aggressive breast cancer cells. *Cancer Res*. 2006;66:5648-5655

56. Greuber EK, Smith-Pearson P, Wang J, Pendergast AM. Role of abl family kinases in cancer: From leukaemia to solid tumours. *Nat Rev Cancer*. 2013;13:559-571

57. Gonzalez-Angulo AM, Hennessy BT, Meric-Bernstam F, Sahin A, Liu W, Ju Z, Carey MS, Myhre S, Speers C, Deng L, Broaddus R, Lluch A, Aparicio S, Brown P, Pusztai L, Symmans WF, Alsner J, Overgaard J, Borresen-Dale AL, Hortobagyi GN, Coombes KR, Mills GB. Functional proteomics can define prognosis and predict pathologic complete response in patients with breast cancer. *Clin Proteomics*. 2011;8:11

58. Henjes F, Bender C, von der Heyde S, Braun L, Mannsperger HA, Schmidt C, Wiemann S, Hasmann M, Aulmann S, Beissbarth T, Korf U. Strong egfr signaling in cell line models of erbb2-amplified breast cancer attenuates response towards erbb2-targeting drugs. *Oncogenesis*. 2012;1:e16

59. Tanos B, Pendergast AM. Abl tyrosine kinase regulates endocytosis of the epidermal growth factor receptor. *J Biol Chem*. 2006;281:32714-32723

60. Thu VT, Kim HK, Ha SH, Yoo JY, Park WS, Kim N, Oh GT, Han J. Glutathione peroxidase 1 protects mitochondria against hypoxia/reoxygenation damage in mouse hearts. *Pflugers Arch*. 2010;460:55-68

61. Gao J, Xiong Y, Ho YS, Liu X, Chua CC, Xu X, Wang H, Hamdy R, Chua BH. Glutathione peroxidase 1-deficient mice are more susceptible to doxorubicin-induced cardiotoxicity. *Biochim Biophys Acta*. 2008;1783:2020-2029

62. Esposito LA, Kokoszka JE, Waymire KG, Cottrell B, MacGregor GR, Wallace DC. Mitochondrial oxidative stress in mice lacking the glutathione peroxidase-1 gene. *Free Radic Biol Med*. 2000;28:754-766

63. Reeves MA, Hoffmann PR. The human selenoproteome: Recent insights into functions and regulation. *Cell Mol Life Sci*. 2009;66:2457-2478

64. Reeves MA, Bellinger FP, Berry MJ. The neuroprotective functions of selenoprotein m and its role in cytosolic calcium regulation. *Antioxid Redox Signal*. 2010;12:809-818

65. Hoffmann FW, Hashimoto AS, Lee BC, Rose AH, Shohet RV, Hoffmann PR. Specific antioxidant selenoproteins are induced in the heart during hypertrophy. *Arch Biochem Biophys*. 2011;512:38-44

66. Brasher BB, Van Etten RA. C-abl has high intrinsic tyrosine kinase activity that is stimulated by mutation of the src homology 3 domain and by autophosphorylation at two distinct regulatory tyrosines. *J Biol Chem*. 2000;275:35631-35637

67. Marcotte R, Zhou L, Kim H, Roskelly CD, Muller WJ. C-src associates with erbb2 through an interaction between catalytic domains and confers enhanced transforming potential. *Mol Cell Biol*. 2009;29:5858-5871

68. Bersell K, Arab S, Haring B, Kuhn B. Neuregulin1/erbb4 signaling induces cardiomyocyte proliferation and repair of heart injury. *Cell*. 2009;138:257-270

69. Tidcombe H, Jackson-Fisher A, Mathers K, Stern DF, Gassmann M, Golding JP. Neural and mammary gland defects in erbb4 knockout mice genetically rescued from embryonic lethality. *Proc Natl Acad Sci U S A*. 2003;100:8281-8286

70. Colicelli J. Abl tyrosine kinases: Evolution of function, regulation, and specificity. *Sci Signal*. 2010;3:re6

71. Olayioye MA, Neve RM, Lane HA, Hynes NE. The erbb signaling network: Receptor heterodimerization in development and cancer. *EMBO J*. 2000;19:3159-3167

72. Wang Z, Zhang L, Yeung TK, Chen X. Endocytosis deficiency of epidermal growth factor (egf) receptor-erbb2 heterodimers in response to egf stimulation. *Mol Biol Cell*. 1999;10:1621-1636

73. Plattner R, Kadlec L, DeMali KA, Kazlauskas A, Pendergast AM. C-abl is activated by growth factors and src family kinases and has a role in the cellular response to pdgf. *Genes Dev*. 1999;13:2400-2411

74. Tanis KQ, Veach D, Duewel HS, Bornmann WG, Koleske AJ. Two distinct phosphorylation pathways have additive effects on abl family kinase activation. *Mol Cell Biol*. 2003;23:3884-3896

75. Ma J, Wang Y, Zheng D, Wei M, Xu H, Peng T. Rac1 signalling mediates doxorubicin-induced cardiotoxicity through both reactive oxygen species-dependent and -independent pathways. *Cardiovasc Res*. 2013;97:77-87

76. Watkins SJ, Borthwick GM, Arthur HM. The h9c2 cell line and primary neonatal cardiomyocyte cells show similar hypertrophic responses in vitro. *In Vitro Cell Dev Biol Anim*. 2011;47:125-131

77. Raskin AM, Hoshijima M, Swanson E, McCulloch AD, Omens JH. Hypertrophic gene expression induced by chronic stretch of excised mouse heart muscle. *Mol Cell Biomech*. 2009;6:145-159

78. Deng XF, Rokosh DG, Simpson PC. Autonomous and growth factor-induced hypertrophy in cultured neonatal mouse cardiac myocytes. Comparison with rat. *Circ Res*. 2000;87:781-788

79. Naga Prasad SV, Duan ZH, Gupta MK, Surampudi VS, Volinia S, Calin GA, Liu CG, Kotwal A, Moravec CS, Starling RC, Perez DM, Sen S, Wu Q, Plow EF, Croce CM, Karnik S. Unique microrna profile in end-stage heart failure indicates alterations in specific cardiovascular signaling networks. *J Biol Chem*. 2009;284:27487-27499

80. Saad SY, Alkharfy KM, Arafah MM. Cardiotoxic effects of arsenic trioxide/imatinib mesilate combination in rats. *J Pharm Pharmacol*. 2006;58:567-573

81. Qiu Z, Cang Y, Goff SP. C-abl tyrosine kinase regulates cardiac growth and development. *Proc Natl Acad Sci U S A*. 2010;107:1136-1141

82. Chislock EM, Ring C, Pendergast AM. Abl kinases are required for vascular function, tie2 expression, and angiopoietin-1-mediated survival. *Proc Natl Acad Sci U S A*. 2013;110:12432-12437

83. Hasinoff BB, Patel D. The lack of target specificity of small molecule anticancer kinase inhibitors is correlated with their ability to damage myocytes in vitro. *Toxicol Appl Pharmacol*. 2010;249:132-139

84. Wolf A, Couttet P, Dong M, Grenet O, Heron M, Junker U, Laengle U, Ledieu D, Marrer E, Nussher A, Persohn E, Pognan F, Riviere GJ, Roth DR, Trendelenburg C, Tsao J, Roman D. Imatinib does not induce cardiotoxicity at clinically relevant concentrations in preclinical studies. *Leuk Res*. 2010;34:1180-1188

85. Hu W, Lu S, McAlpine I, Jamieson JD, Lee DU, Marroquin LD, Heyen JR, Jessen BA. Mechanistic investigation of imatinib-induced cardiac toxicity and the involvement of c-abl kinase. *Toxicol Sci*. 2012;129:188-199

86. Kerkela R, Grazette L, Yacobi R, Iliescu C, Patten R, Beahm C, Walters B, Shevtsov S, Pesant S, Clubb FJ, Rosenzweig A, Salomon RN, Van Etten RA, Alroy J, Durand JB, Force T. Cardiotoxicity of the cancer therapeutic agent imatinib mesylate. *Nat Med*. 2006;12:908-916

87. Wang GW, Schuschke DA, Kang YJ. Metallothionein-overexpressing neonatal mouse cardiomyocytes are resistant to h2o2 toxicity. *Am J Physiol*. 1999;276:H167-175

88. Gabrielson KL, Hogue BA, Bohr VA, Cardounel AJ, Nakajima W, Kofler J, Zweier JL, Rodriguez ER, Martin LJ, de Souza-Pinto NC, Bressler J. Mitochondrial toxin 3-nitropropionic acid induces cardiac and neurotoxicity differentially in mice. *Am J Pathol*. 2001;159:1507-1520

89. Fan GC, Zhou X, Wang X, Song G, Qian J, Nicolaou P, Chen G, Ren X, Kranias EG. Heat shock protein 20 interacting with phosphorylated akt reduces doxorubicin-triggered oxidative stress and cardiotoxicity. *Circ Res*. 2008;103:1270-1279

90. Aon MA, Cortassa S, O'Rourke B. Redox-optimized ros balance: A unifying hypothesis. *Biochim Biophys Acta*. 2010;1797:865-877

91. Tahara EB, Navarete FD, Kowaltowski AJ. Tissue-, substrate-, and site-specific characteristics of mitochondrial reactive oxygen species generation. *Free Radic Biol Med*. 2009;46:1283-1297

92. Dikalov SI, Kirilyuk IA, Voinov M, Grigor'ev IA. Epr detection of cellular and mitochondrial superoxide using cyclic hydroxylamines. *Free Radic Res*. 2011;45:417-430

93. Emanuel SL, Hughes TV, Adams M, Rugg CA, Fuentes-Pesquera A, Connolly PJ, Pandey N, Moreno-Mazza S, Butler J, Borowski V, Middleton SA, Gruninger RH, Story JR, Napier C, Hollister B, Greenberger LM. Cellular and in vivo activity of jnj-28871063, a nonquinazoline pan-erbb kinase inhibitor that crosses the blood-brain barrier and displays efficacy against intracranial tumors. *Mol Pharmacol*. 2008;73:338-348

94. Gorlick R, Kolb EA, Houghton PJ, Morton CL, Phelps D, Schaiquevich P, Stewart C, Keir ST, Lock R, Carol H, Reynolds CP, Maris JM, Wu J, Smith MA. Initial testing (stage 1) of lapatinib by the pediatric preclinical testing program. *Pediatr Blood Cancer*. 2009;53:594-598

95. Liang Q, Carlson EC, Donthi RV, Kralik PM, Shen X, Epstein PN. Overexpression of metallothionein reduces diabetic cardiomyopathy. *Diabetes*. 2002;51:174-181

96. Patel N, Barrientos A, Landgraf R. The growth factor receptor erbb2 regulates mitochondrial activity on a signaling time scale. *J Biol Chem*. 2013;288:35253-35265

97. Fagard R. Athlete's heart. *Heart*. 2003;89:1455-1461

98. Abel ED, Doenst T. Mitochondrial adaptations to physiological vs. Pathological cardiac hypertrophy. *Cardiovasc Res*. 2011;90:234-242

99. Goffart S, von Kleist-Retzow JC, Wiesner RJ. Regulation of mitochondrial proliferation in the heart: Power-plant failure contributes to cardiac failure in hypertrophy. *Cardiovasc Res*. 2004;64:198-207

100. Hunter JJ, Chien KR. Signaling pathways for cardiac hypertrophy and failure. *N Engl J Med*. 1999;341:1276-1283

101. Grossman W, Jones D, McLaurin LP. Wall stress and patterns of hypertrophy in the human left ventricle. *J Clin Invest*. 1975;56:56-64

102. Jornayvaz FR, Shulman GI. Regulation of mitochondrial biogenesis. *Essays Biochem*. 2010;47:69-84

103. Kelly DP, Scarpulla RC. Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes Dev*. 2004;18:357-368

104. Schreiber SN, Emter R, Hock MB, Knutti D, Cardenas J, Podvinec M, Oakeley EJ, Kralli A. The estrogen-related receptor alpha (erralpha) functions in ppargamma coactivator 1alpha (pgc-1alpha)-induced mitochondrial biogenesis. *Proc Natl Acad Sci U S A*. 2004;101:6472-6477

105. Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS. Calcium, atp, and ros: A mitochondrial love-hate triangle. *Am J Physiol Cell Physiol*. 2004;287:C817-833

106. Brand MD, Nicholls DG. Assessing mitochondrial dysfunction in cells. *Biochem J*. 2011;435:297-312

107. Ly JD, Grubb DR, Lawen A. The mitochondrial membrane potential (deltapsi(m)) in apoptosis; an update. *Apoptosis*. 2003;8:115-128

108. Perry SW, Norman JP, Barbieri J, Brown EB, Gelbard HA. Mitochondrial membrane potential probes and the proton gradient: A practical usage guide. *Biotechniques*. 2011;50:98-115

109. Kohlhaas M, Maack C. Calcium release microdomains and mitochondria. *Cardiovasc Res*. 2013;98:259-268

110. Ichas F, Jouaville LS, Mazat JP. Mitochondria are excitable organelles capable of generating and conveying electrical and calcium signals. *Cell*. 1997;89:1145-1153

111. Gibbs C. Respiratory control in normal and hypertrophic hearts. *Cardiovasc Res*. 1999;42:567-570

112. Jin H, Nass RD, Joudrey PJ, Lyon AR, Chemaly ER, Rapti K, Akar FG. Altered spatiotemporal dynamics of the mitochondrial membrane potential in the hypertrophied heart. *Biophys J*. 2010;98:2063-2071

113. Slodzinski MK, Aon MA, O'Rourke B. Glutathione oxidation as a trigger of mitochondrial depolarization and oscillation in intact hearts. *J Mol Cell Cardiol*. 2008;45:650-660

114. Viola HM, Hool LC. Targeting calcium and the mitochondria in prevention of pathology in the heart. *Curr Drug Targets*. 2011;12:748-760

115. Di Lisa F, Menabo R, Canton M, Barile M, Bernardi P. Opening of the mitochondrial permeability transition pore causes depletion of mitochondrial and cytosolic nad+ and is a causative event in the death of myocytes in postischemic reperfusion of the heart. *J Biol Chem*. 2001;276:2571-2575

116. Das S, Wong R, Rajapakse N, Murphy E, Steenbergen C. Glycogen synthase kinase 3 inhibition slows mitochondrial adenine nucleotide transport and regulates voltage-dependent anion channel phosphorylation. *Circ Res*. 2008;103:983-991

117. Imahashi K, Schneider MD, Steenbergen C, Murphy E. Transgenic expression of bcl-2 modulates energy metabolism, prevents cytosolic acidification during ischemia, and reduces ischemia/reperfusion injury. *Circ Res*. 2004;95:734-741

Curriculum vitae

Frances R. Belmonte, M.S.
(757) 344-7003
fbelmon2@jhmi.edu
929 St. Paul St. #3F
Baltimore, MD 21202

Birth date: November 23, 1984
Birth location: Wise, VA

EDUCATION

Ph.D. Candidate, Molecular and Translational Toxicology, Expected May 2014
Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
Dissertation: Using a Model of Cardiac-Specific Over-Expression of Epidermal Growth Factor Receptor 2 (ErbB2) to Study Redox Signaling, Oxidant Stress, and Mitochondrial Function

Master of Science in Environmental Health Sciences, Concentration in Toxicology, September 2009
Department of Environmental Medicine, New York University, New York, NY
Thesis title: Differential Toxicity and Gene Expression of Atlantic tomcod (*Microgadus tomcod*) from Two Populations to Polycyclic Aromatic Hydrocarbons, a Polychlorinated Biphenyl Congener, Chromium, and their Binary Combinations

Bachelor of Science, Biochemistry, December 2005
Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA

RESEARCH EXPERIENCE

Graduate Research Assistant, Department of Environmental Health Sciences, Johns Hopkins University, Baltimore, MD, August 2009-Present
A murine model with cardiomyocyte-specific over-expression of epidermal growth factor receptor 2 (ErbB2) that has hypertrophy without heart failure was investigated to study: 1) altered redox-signaling that has downstream effects on oxidant stress and 2) differences in mitochondrial function compared to wild type that have yet to be fully characterized using the following skills and techniques:

- Performed electrocardiography (EKG) to identify the wild type from the ErbB2 transgenic animals, which have distinctive EKG tracings that are characteristic of cardiac hypertrophy
- Characterized proteins and genes that are activated by the ErbB2 signaling pathway via molecular techniques such as immunoblotting, co-immunoprecipitation, DNA/RNA isolations, PCR, and quantitative PCR
- Measured reactive oxygen species (ROS) and oxidative stress via electron paramagnetic resonance and Amplex Red assays as well as various ROS detection

dyes using heart tissue and mitochondria isolated from hearts via differential centrifugation
- Utilized enzymatic activity assays for glutathione peroxidase, glutathione reductase, and thioredoxin reductase on heart tissue and mitchondria
- Administered doxorubicin and lapatinib in different settings to assess molecular changes
- Performed *in vitro* cell culture studies on HEK293 and H9c2 cell lines and neonatal cardiomyocytes isolated from wild type and ErbB2 transgenic hearts
- Isolated cardiac mitochondria via differential centrifugation for respiration and mitochondrial function studies
- Transfected HEK293 and H9c2 cells to transiently overexpress ErbB2 and performed siRNA-mediated assays to knockdown ErbB2 in H9c2 cells

Graduate Research Assistant, Department of Environmental Medicine, New York University, New York, NY, August 2007-May 2009
The Atlantic tomcod (*Microgadus tomcod*) has been used as a fish model to assess exposure effects from Hudson River contaminants. A toxicity study was conducted on two populations of Atlantic tomcod fish that were exposed to various polyaromatic hydrocarbons, a polychlorinated biphenyl congener, hexavalent chromium (Cr (VI)), or a mixture of one of the compounds with Cr (VI). The following skills were performed to assess the exposure effects from the contaminants:
- Extracted RNA from fish embryos exposed to the compounds for real-time reverse transcription- polymerase chain reaction (RT-PCR) to assess differences in gene expression
- Utilized ImageJ software to measure morphological changes caused by the compounds

Research Technician, Duke Human Vaccine Institute, Duke University, Durham, NC, February 2007-August 2007
Approximately 4,500 patient plasma samples were measured for various cytokines and the following skills were employed:
- Quantified various cytokines in the plasma samples via Multiplex bead-based immunoassays (Luminex)
- Maintained a large database for the plasma samples

Research Technician III, Division of Nephrology and Hypertension, University of North Carolina-Chapel Hill, Chapel Hill, NC, March 2006-February 2007
Antineutrophil cytoplasmic autoantibody (ANCA) -mediated glomerulonephritis patient autoantibody reactivity to proteinase-3 (PR3) antibodies was assessed by the following techniques:
- Transfected HEK293 cells to produce recombinant PR3 proteins
- Purified recomibinant proteins on HisTrap affinity columns and identified the proteins via immunoblotting

TEACHING EXPERIENCE

Teaching Assistant, Graduate Level Course-Environmental Toxicological Pathology, Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, March 2012-May 2012

- Collaborated with the instructor to construct the exams based on specific questions and their corresponding answers that were developed by the students
- Assisted with the organization of homework assignments and course material
- Hosted guest lecturers

AWARDS & FUNDING

- Delta Omega Scientific Poster Competition, 2nd place, Delta Omega Alpha Chapter, Johns Hopkins Bloomberg School of Public Health, February 2013
- National Capital Area Chapter of the Society of Toxicology Bern Schwetz Award, Honorable Mention, March 2012
- National Institute of Environmental Health Sciences (NIEHS)-supported training grant, August 2009-Present

PUBLICATIONS

- Polina Sysa-Shah, Yi Xu, Xin Guo, **Frances Belmonte**, Byunghak Kang, Djahida Bedja, Scott Pin, Noriko Tsuchiya, and Kathleen Gabrielson. Cardiac-Specific Over-Expression of Epidermal Growth Factor Receptor 2 (ErbB2) Induces Pro-Survival Pathways and Hypertrophic Cardiomyopathy in Mice. PLoS One, 2012; 7(8): e42805.
- Peter Hewins, **Frances Belmonte**, J. Charles Jennette, Ronald J. Falk, Gloria A. Preston. Longitudinal Studies of Patients with ANCA Vasculitis Demonstrate Concurrent Reactivity to Complementary PR3 Protein Segments cPR3m and cPR3C and with no Reactivity to cPR3N. Autoimmunity, 2011; 44(2): 98-106.

ABSTRACTS

- "Chronic Over-Expression of Epidermal Growth Factor Receptor 2 (ErbB2) in the Heart Alters Redox-Sensitive Molecules that Modulate Reactive Oxygen Species (ROS) Formation," **Frances Belmonte**, Sam Das, Polina Sysa-Shah, Yi Xu, Vidhya Sivakumaran, Yongqing Zhang, Nazareno Paolocci, Charles Steenbergen, Kathleen Gabrielson. Society of Toxicology Conference 2013, San Antonio, TX, March 10-14, 2013.
- "Chronic Over-Expression of Receptor Tyrosine Kinase ErbB2 in the Heart Affects Mitochondrial Function and Oxidative Stress," **Frances Belmonte**, Polina Sysa-Shah, Yi Xu, Xin Guo, Sam Das, Vidhya Sivakumaran, Djahida Bedja, Nazareno Paolocci, Charles Steenbergen, Kathleen Gabrielson. Society of Toxicology Conference 2012, San Francisco, CA, March 11-15, 2012.
- "Chronic Over-Expression of Receptor Tyrosine Kinase ErbB2 Induces Cellular Oxidative Stress in the Hearts of Mice," **Frances Belmonte**, Polina Sysa-Shah, Xin Guo, Sam Das, Scott Pin, Kathleen Gabrielson. Society of Toxicology Conference 2011, Washington, D.C., March 6-10, 2011.

PROFESSIONAL MEMBERSHIPS

- Society of Toxicology, December 2010-Present

- National Capital Area Chapter of the Society of Toxicology, January 2012-Present

COMMUNITY SERVICE

- Baltimore Flute Choir, Baltimore, MD, September 2010-Present
- The New York Botanical Garden, Bronx, NY, September 2007-November 2007
- Toxic Free NC, Raleigh, NC, February 2006-August 2007